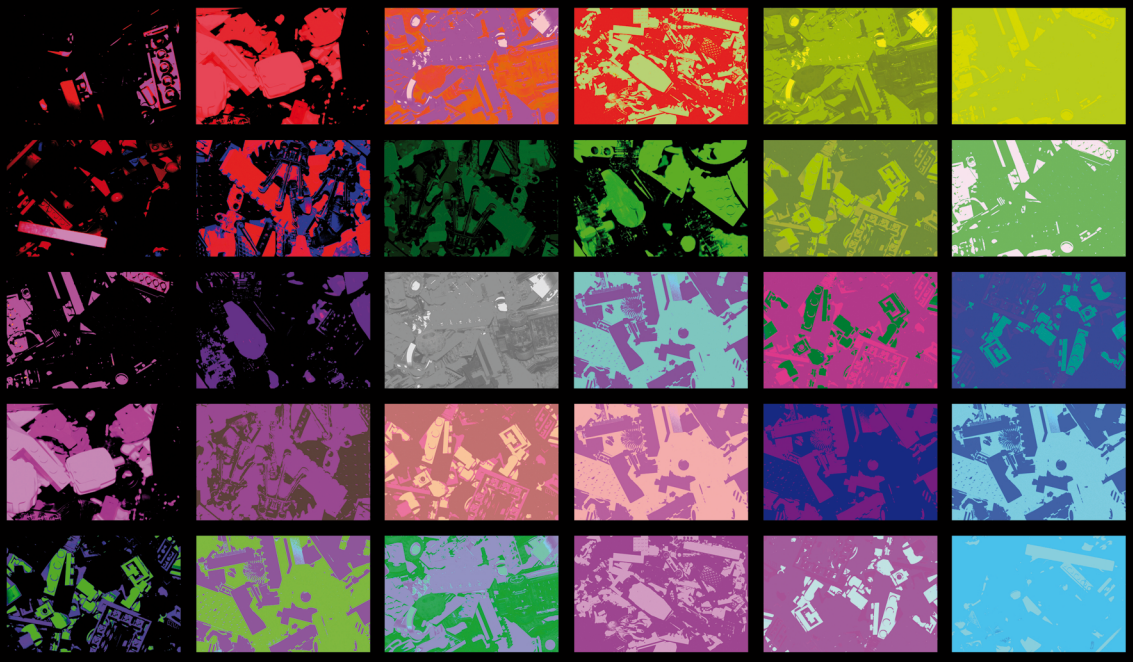
Cross-talk of inflammatory pathways for pathogen recognition



Gerben Ferwerda

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Colofon

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Cross-talk of inflammatory pathways for pathogen recognition

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Chapter 1

Introduction and outline of the thesis

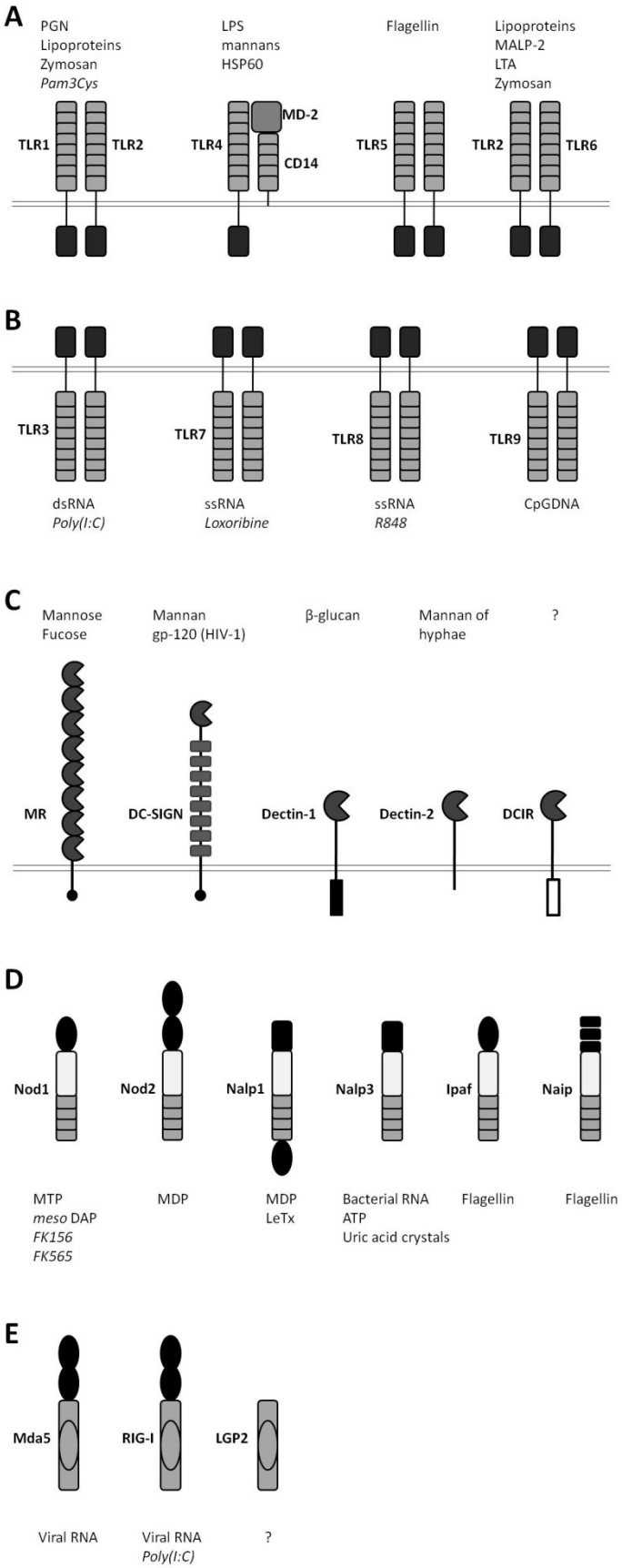
Introduction

The interplay between host and microbes has fascinated many scientists and has led to the discovery of vaccination and antibiotics, both crucial in the history of mankind. When Robert Koch (1843-1910), Louis Pasteur (1822-1895) and Ferdinand J. Cohn (1828 –1898) defined the fundaments for microbiology, Ilya I. Metchnikov (1845-1918) laid a solid fundament for immunology by the discovery of phagocytosis by specialized mobile immune cells he named phagocytes. Phagocytes have an inherited capability to protect the host against invading microorganisms, and this is called the innate immune response. How the phagocytes could recognize pathogens has been a riddle for almost a century.

A conceptual and visionary breakthrough was proposed by Charles A. Janeway, Jr. (1943-2003) twenty years ago, when he proposed that cells of the innate immune system should possess receptors for recognition of structures unique for pathogens, in order to discriminate between self and non-self. He termed these receptors *pattern recognition receptors* (PRRs). It was however not until 1996 when Jules A. Hoffmann and his colleagues identified *Drosophila* Toll molecule as an essential receptor on the surface of the haematocyte, mediating immunity to fungal infection, which it achieved by the activation of the synthesis of antimicrobial peptides [1]. One year later, the first homologues of *Toll* were found in humans and named Toll-like receptors (TLRs). Ligation of these receptors leads to secretion of cytokines and activation of the antimicrobial host defense [2,3].

During the past decade, several classes of PRRs have been identified in addition to TLRs, among which C-type lectins, Nod-like receptors, and RigI-helicases. Intense research efforts have identified the ligands for most of them (Fig 1). Insight into the signal pathways activated by the engagement of these receptors and the secretion of cytokines by cells of the innate immune system soon identified modulatory effects of PRR activation on the adaptive immune responses, and led to the concept of a 'bridge' between innate and adaptive immune system (Fig 2) [2,4]. One of the most important questions raised, however, was how apparently a 'few' receptors could induce specific immune responses against a legion of pathogens. Because pathogens express a series of ligands that can be recognized by different PRRs, it is the simultaneous activation of certain PRR combinations by each invading pathogen that is now believed to bring specificity to the immune response against pathogens [5]. The cross-talk between these pathways leads to stimulation or inhibition of immunological responses, and is crucial for the outcome of an infection.

Because the innate immune system is known to play an important role in infectious, autoimmune and autoinflammatory diseases, insight into the activation of the innate immune response leads to understanding the pathophysiological mechanisms of these diseases and eventually to development of new therapeutic strategies. Indeed, genetic mutations in genes coding for PRRs are linked to several diseases. Mutations in the Nod-like receptors (NLRs) genes are generally associated with autoinflammatory disease.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Lipoproteins
Zymosan
Pam3Cys
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mannans
HSP60
Flagellin
Lipoproteins
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Zymosan
TLR1
TLR2
TLR4
MD-2
CD14
TLR5
TLR2
TLR6
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TLR7
TLR8
TLR9
dsRNA
Poly(I:C)
ssRNA
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ssRNA
R848
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Fucose
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gp-120 (HIV-1)
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MR
DC-SIGN
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D
Nod1
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MTP
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FK156
FK565
MDP
MDP
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ATP
Uric acid crystals
Flagellin
Flagellin
E
Mda5
RIG-I
LGP2
Viral RNA
Viral RNA
Poly(I:C)
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Figure 1. Pattern recognition receptors and their ligands. There are four different classes of pattern recognition receptors known: **Toll-like receptors** expressed on the cell surface (A) and expressed in the phagosome (B) containing a recognition domain of leucine rich repeats and a TLR/IL-1 receptor (TIR) domain; **C-type lectin receptors** expressed on the cell surface and in the phagosome (C) bearing a common carbohydrate recognition domain; **Nod-like receptors** expressed in the cytosol (D) containing a nucleotide oligomerization domain and mostly a leucine rich recognition domain; and **RigI helicases** (E) with a helicase domain and a C-terminal repression domain.

Recently, mutations in NALP3 have been shown to be the cause of Muckle-Wells syndrome and Familial Cold Urticaria [6], while mutations in Nod2 are associated with Crohn's disease and Blau syndrome [7-9]. In infectious disease, polymorphisms in PRR genes have been linked to a higher susceptibility for specific pathogens, e.g. polymorphisms in TLR4 have been linked to Gram-negative sepsis [10], malaria [11], *Candida* infections [12] and *Aspergillus fumigatus* infection [13], TLR2 polymorphisms to increased susceptibility to mycobacterial infections [14], while TLR5 have been linked to *Legionella* pneumonia [15]. Total deficiency of the TLR pathways in patients with defects in MyD88 or IRAK4 leads to severe infections with streptococci, staphylococci or *Pseudomonas*, with 30% of the patients succumbing to infection before they reach 20 years of age [16,17].

Outline of the thesis

The aim of this thesis is to study the interaction between the signaling pathways of different classes of pattern recognition receptors (PRRs), in order to understand the mechanisms through which initial recognition of microbial ligands lead to a fully developed innate immune response. To achieve this aim, we will use production of cytokines as read-out for the activated signal pathways, as cytokines both activate innate host defense and modulate adaptive immunity.

In the first part of the thesis, I will focus on the recognition pathways of *Candida albicans*, the most important human fungal pathogen with an important morbidity and mortality burden on the society. *C. albicans* is currently the fourth most common microorganism in patients with blood stream infection in the USA [18], and the mortality in patients with disseminated candidiasis is still 30 to 40%, despite the availability of new antifungal drugs [19]. In addition, vulvovaginal candidiasis (VVC) causes major morbidity, with 5% of women suffering from recurrent VVC. We and others have previously shown that TLRs and CLRs are the two most important classes of receptors involved in *C. albicans* recognition, but little is known regarding the cross-talk between these receptors during fungal recognition. We will focus on the interaction between TLR2, TLR4 and dectin-1, as they are known to recognize *C. albicans*, their ligands have been identified, and their signal pathways have been well studied. These three receptors are also known to be important in the host defense against *Candida* and they may be relevant in the development of future new therapeutic strategies.

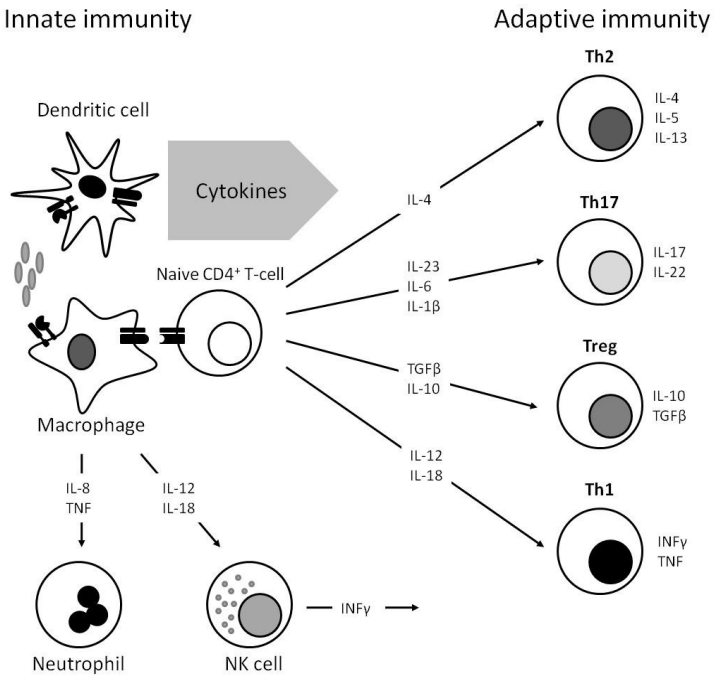

Figure 2. Pattern recognition receptors regulate adaptive immune responses. Dendritic cells and macrophages (antigen presenting cells (APC)), neutrophils and natural killer cells are the major innate immune cells, whereas the adaptive immune cells consist of B-cells and T-cells. Pattern recognition receptors are abundantly expressed on APCs. When microbial ligands bind to PRRs, signal pathways are activated and cytokines secreted. After phagocytosis, the microbes are degraded in the phagolysosome and presented on the MHCII. During this stage, also the intracellular PRRs signal pathways are also activated. Expression of costimulatory molecules on the membrane of the antigen presenting cell is induced by PRR ligation. The antigen/MHCII complex and costimulatory molecules on the APC will lead to activation of the T-cell receptor and proliferation of the naïve T-cell. The cytokines produced by the APCs are crucial and determine the differentiation of the T*helper* cell.

How do immune cells respond when two classes of PRRs are stimulated simultaneously? This question is studied in **Chapter 2** by using specific ligands for TLR2, TLR4 and dectin-1. In **Chapter 3**, the nature of the interaction between these receptors will be further investigated in an *in-vitro* model, in order to obtain more insight in the amplification mechanism at the level of the intracellular signal pathways of both classes of PRRs. The dectin-1 signal pathway is activated by β-glucan, a major cell wall component of *Candida albicans*, and induces the production of cytokines by macrophages. In **Chapter 4** we will further investigate the effects exerted by activation of this pathway by using a novel screening approach to identify membrane-bound molecules regulated by dectin-1.

The role of dectin-1 and its interaction with TLRs in the pathogenesis of fungal disease is illustrated by the identification of a single nucleotide polymorphism (SNP) in the dectin-1 gene, leading to defective function of the protein, with important consequences for recognition of beta-glucans. In **Chapter 5**, we explore the cellular responses of cells isolated from individuals homozygous for this SNP, and describe the clinical phenotype of these patients.

In **Chapter 6**, we will summarize the current knowledge on the interaction between TLR and CLRs in *Candida albicans* infections, and place these findings into the perspective of the development of new therapeutic strategies, such as vaccination, against *Candida albicans*.

The second part of this thesis will investigate the cross-talk between TLRs and Nod2, a member of the Nod-like receptor (NLR) class of recognition receptors that have been implicated in the immune responses during both autoimmune (e.g. Crohn's disease) and infectious diseases. Crohn's disease is a chronic granulomatous inflammation of the gut, and mutations in the Nod2 gene have been associated with a high incidence of this disease [7,8]. In addition, Nod2 is an intracellular receptor of bacterial peptidoglycans, and thereby might also play a role in the defence against intracellular pathogens. The cross-talk between TLRs and Nod2 will be investigated in primary mononuclear cells isolated from patients with Crohn's disease bearing a mutation in the Nod2 gene, which leads to unresponsiveness to stimulation with the peptidoglycan component muramyl dipeptide (MDP). In **Chapter 7**, primary human mononuclear cells are stimulated with TLR and Nod2 ligands in order to assess the nature of interaction between these two classes of PRRs. Transcriptional and post-transcriptional mechanisms involved in this interaction are presented in **Chapter 8**.

In **Chapter 9 and 10**, we will assess the role of Nod2 as a receptor for mycobacteria, and we will illustrate the consequences of mutations in the Nod2 gene for the innate immune response against *Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis*. The cross-talk between TLRs and Nod2 for the host defence mechanisms to mycobacteria will be highlighted.

In the previous chapters we have investigated the simultaneous stimulation of PRRs as model for invading microorganisms. Because the innate immune cells of the gut are continuously exposed to commensal intestinal microorganisms, suppression of the immune response of the host is desirable. This phenomenon is called tolerance and is thought to be exerted by long-lasting stimulation of pattern recognition receptors by gut commensal flora and downregulation of PRR-signal pathways [20]. When the innate immune system is continuously stimulated by PRR ligands, this might lead to autoinflammatory diseases. In **Chapter 11**, we will use phased stimulation of primary mononuclear cells isolated from patients with mutations in the Nod2 gene, in order to study the cross-talk between TLRs and Nod2 as a model for continuous stimulation of PRR and induction of tolerance.

The interaction between Nod2 and its ligand muramyl dipeptide (MDP) seems to play an important role in the pathogenesis of Crohn's disease. In the previous chapters we investigated the modulatory effect of Nod2 ligation of MDP by Nod2 on TLR signal pathways, but what is the immunomodulatory effect of other muramyl peptides? In **Chapter 12**, we will use various muramyl peptides to stimulate primary cells in combination with TLR ligands to answer this question. Among these compounds are specific ligands for Nod1, a member of the NLR family closely related to Nod2, which will give us the opportunity to obtain more insight into the common and specific activated signalling pathways by both PRRs.
A summary of our findings and conclusions of the thesis are presented in **Chapter 13**.

Reference List

1 Lemaitre,B. *et al.* (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86, 973-983

2 Medzhitov,R. *et al.* (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388, 394-397

3 Poltorak,A. *et al.* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085-2088

4 Takeda,K. *et al.* (2003) Toll-like receptors. *Annu. Rev. Immunol.* 21, 335-376

5 Netea,M.G. *et al.* (2004) Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. *J. Leukoc. Biol.* 75, 749-755

6 Aganna,E. *et al.* (2002) Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. *Arthritis Rheum.* 46, 2445-2452

7 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

8 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

9 Miceli-Richard,C. *et al.* (2001) CARD15 mutations in Blau syndrome. *Nat. Genet.* 29, 19-20

10 Agnese,D.M. *et al.* (2002) Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J. Infect. Dis.* 186, 1522-1525

11 Mockenhaupt,F.P. *et al.* (2006) Toll-like receptor (TLR) polymorphisms in African children: Common TLR-4 variants predispose to severe malaria. *Proc. Natl. Acad. Sci. U. S. A* 103, 177-182

12 Van der Graaf,C.A. *et al.* (2006) Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for Candida bloodstream infection. *Eur. Cytokine Netw.* 17, 29-34

13 Bochud,P.Y. *et al.* (2008) Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *N. Engl. J. Med.* 359, 1766-1777

14 Kang,T.J. and Chae,G.T. (2001) Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. *FEMS Immunol. Med. Microbiol.* 31, 53-58

15 Hawn,T.R. *et al.* (2003) A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J. Exp. Med.* 198, 1563-1572

16 Picard,C. *et al.* (2003) Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science* 299, 2076-2079

17 von Bernuth H. *et al.* (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321, 691-696

18 Edmond,M.B. *et al.* (1999) Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Infect. Dis.* 29, 239-244

19 Pfaller,M.A. and Diekema,D.J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20, 133-163

20 Rakoff-Nahoum,S. *et al.* (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229-241

Part I

Receptor cross-talk for recognition of fungal pathogens: TLRs and CLRs

Chapter 2

Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages

Gerben Ferwerda[†], Friederike Meyer-Wentrup[†], Bart Jan Kullberg, Mihai G. Netea and Gosse J. Adema

[†] These two authors contributed equally to the study.

Cellular Microbiology. 2008 Oct;10(10):2058-66

Abstract

The β-glucan receptor dectin-1 and Toll-like receptors TLR2 and TLR4 are the main receptors for recognition of *Candida albicans* by the innate immune system. It has been reported that dectin-1 amplifies TLR2-dependent induction of cytokines in mouse models. In the present study we hypothesized that dectin-1 has potent synergistic effects with both TLR2 and TLR4 in human PBMCs and macrophages. Human PBMCs and monocyte-derived macrophages were stimulated with curdlan, a linear β-1,3-glucan-polymer derived from *Alcaligenes faecalis* with specific ligand affinity for dectin-1, in combination with the synthetic TLR2 ligand Pam3Cys and the ultrapure TLR4 ligand LPS. TNFα and IL-10 production was measured in the supernatants with ELISA. Curdlan is a specific dectin-1 ligand without TLR2- or TLR4-stimulating properties. Human primary monocytes and macrophages express dectin-1 on the cell membrane. Stimulation of human PBMCs with curdlan in combination with Pam3Cys or LPS leads to synergistic increase in TNFα production that was inhibited by GE2, a neutralizing dectin-1 antibody. Dectin-1-dependent synergy between curdlan and TLR agonists was also apparent in human monocyte-derived macrophages. Conclusively, dectin-1 synergizes with both TLR2 and TLR4 pathways for the production of TNFα in human primary PBMCs and in monocyte-derived macrophages.

Introduction

Candida albicans forms part of the commensal microbial flora of humans. However, in some cases *C. albicans* encounter can lead to inflammation and infection of mucosal surfaces (mucocutaneous candidiasis), or even to invasion of tissues or bloodstream (disseminated candidiasis), especially in the immunocompromised host. Despite the availability of effective novel antifungal therapies, bloodstream infections with C. albicans are still responsible for a high mortality up to 40% [1].

Cells involved in first line defense against *C. albicans*, like neutrophils, monocytes and macrophages, are important for activation and regulation of innate immune responses. These cells express pattern-recognition receptors (PRRs) on their cell membrane, which recognize pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms. PRR engagement leads to induction of cytokine, chemokine and antimicrobial peptide production, activation of oxygen radical release and eventually elimination of the invading pathogen [2]. The best known and characterized classes of PRRs are the Toll-like receptors (TLRs) [3] and C-type lectin receptors (CLRs) [4,5]. Both are involved in recognition of *C. albicans* [6-8] and bind to different structures of the fungal surface. The outer layer of the *C. albicans* cell wall is enriched with mannoproteins. TLR2 recognizes phospholipomannan [9], while TLR4 binds to O-linked mannosyl residues [10]. N-linked mannosyl residues are detected by a CLR, the macrophage mannose receptor (MR) [11]. The inner layer of C. albicans consists of β-glucans, which are recognized by another CLR, dectin-1 [12-15]. Each of these receptors induces specific responses after exposure to *C. albicans*. Their importance in the antifungal host defense is underlined by a higher susceptibility for *C. albicans* infections in dectin-1- and TLR4-deficient mice [16,17] and a higher resistance against *C. albicans* in TLR2-deficient mice [18]. TLR2 stimulation with *C. albicans* induces mainly an anti-inflammatory cytokine profile [19] and affects regulatory T-cell function [20], whereas stimulation of TLR4 leads to a pro-inflammatory cytokine response [21]. Interestingly, it has been shown that CLRs and TLRs can interact with each other: in murine macrophages and dendritic cells the β-glucan receptor dectin-1 amplifies TLR2-mediated cytokine release [22,23]. A picture emerges in which cross-talk of TLRs and CLRs leads to a specific innate immune response against *C. albicans* [24,25].

To get more insight into the interactions of PRRs involved in recognizing *C. albicans*, we developed an in vitro model in which human PBMCs and monocyte-derived macrophages were stimulated with pure receptor ligands. We hypothesized that in addition to its synergistic effects on TLR2-mediated signaling, dectin-1 may also amplify the effects of TLR4 triggering. The data regarding TLR/CLR interactions reported in literature until now have been obtained mainly with cell lines or mouse macrophages. Our results demonstrate that in human primary monocytes and macrophages dectin-1 synergizes with both TLR2 and TLR4.

Results

Dectin-1, TLR2 and TLR4 mediate C. albicans-induced cytokine production by human PBMCs

To examine the contribution of dectin-1 and TLRs in *C. albicans*-induced cytokine production, we stimulated human PBMCs with heat-killed *C. albicans* and inhibited different receptors involved in *C. albicans* recognition (Fig. 1). Inhibition of TLR2 with a blocking antibody resulted in a reduction of 55 ± 10% in TNFα secretion. When TLR4 was inhibited, TNFα production was decreased by 26 ± 21%. Dectin-1 inhibition with glucan phosphate inhibited TNFα production by 80 ± 11%. The cumulative reduction in TNFα production adding up to more than 100% when inhibiting dectin-1 and TLR2/4 could possibly be explained by the presence of synergistic effects between these receptors. In case of inhibition of one pathway, both the specific effects of that pathway and the synergistic action on other receptors will be lost.

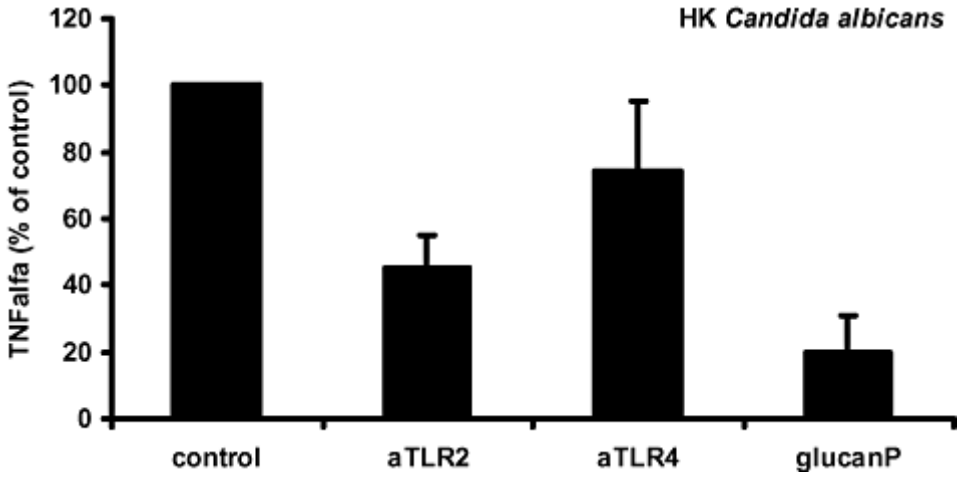

Figure 1. Cytokine induction by heat-killed C. albicans in mononuclear cells is mediated by different classes of receptors. Human PBMCs of five healthy volunteers were incubated with 1 × 10^6 heat-killed (HK) C. albicans after 1 h pre-incubation with anti-TLR2 (10 μg ml^{-1}), isotype-control (10 μg ml^{-1}), TLR4 antagonist (100 ng ml^{-1}) or glucan phosphate (100 μg ml^{-1}). After 24 h incubation at 37°C, TNFα was measured by ELISA in the supernatant. Data are presented as percentage of control (medium stimulated or isotype control) ± SD.

Curdlan is a pure dectin-1 agonist and induces TNFα production in human PBMCs and macrophages

Candida albicans contains dectin-1, TLR2 and TLR4 ligands. To further study the interaction between TLRs and dectin-1, human PBMCs and macrophages were incubated with pure receptor ligands. First, expression of dectin-1 by these human cell populations was determined. FACS analysis showed that monocytes in PBMCs are the dominant cell population expressing dectin-1, and that monocyte-derived macrophages in addition to the MR also have dectin-1 on their cell surface (Fig. 2A and B).

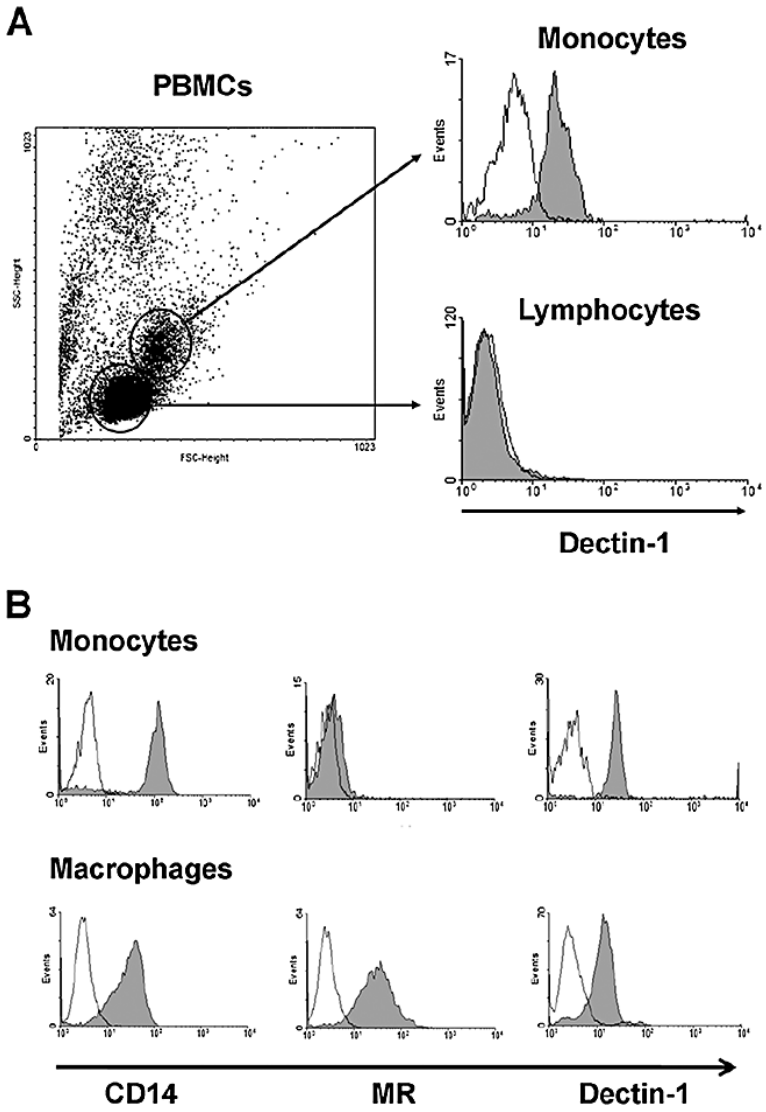

Figure 2. Human monocytes and macrophages express dectin-1 on their cell surface. Human PBMCs were labeled with goat anti-dectin-1 (grey shaded area) or matched isotype control (thin line) and fluorochrome-conjugated secondary antibodies and analyzed by FACS. Gates were set on lymphocyte and monocyte populations according to forward/sideward scatter characteristics. Among the PBMCs only monocytes express dectin-1. There is no expression detectable on the lymphocyte population. Data are representative of three different donors (A). Human monocytes and monocyte-derived macrophages were stained with anti-CD14, anti-Mannose receptor (MR) anti-dectin-1 (grey shaded area) or matched isotype control (thin line) antibodies and fluorochrome-conjugated secondary antibodies and analyzed by FACS. In contrast to monocytes, macrophages express MR. Both cell populations are CD14 positive and express similar levels of dectin-1 on their surface. Data are representative of three different donors (B).

Curdlan is a linear β-1,3-glucan polymer derived from the bacterium *Alcaligenes faecalis* [26] and is a known ligand for the β-glucan receptor in mice [27-29]. We used HEK293 cells transfected with human TLR2 to determine if curdlan had any TLR activating properties. As shown in Fig. 3A, in contrast with zymosan, curdlan did not induce TLR2-mediated IL-8 production in these cells. There was also no difference in TNFα production between TLR4 wild-type and TLR4-deficient murine macrophages (data not shown), indicating that curdlan

is not contaminated with LPS or other TLR4 ligands. When human PBMCs were stimulated with curdlan, a dose-dependent induction of TNFα and to a lesser extent of IL-10 was observed. Human macrophages hardly produced TNFα and little IL-10 in response to dectin-1 triggering (Fig. 3B). The differential capacity of monocytes (the dectin-1 expressing cells in PBMCs) and macrophages in their production of pro-inflammatory cytokines is a well-known phenomenon [26].

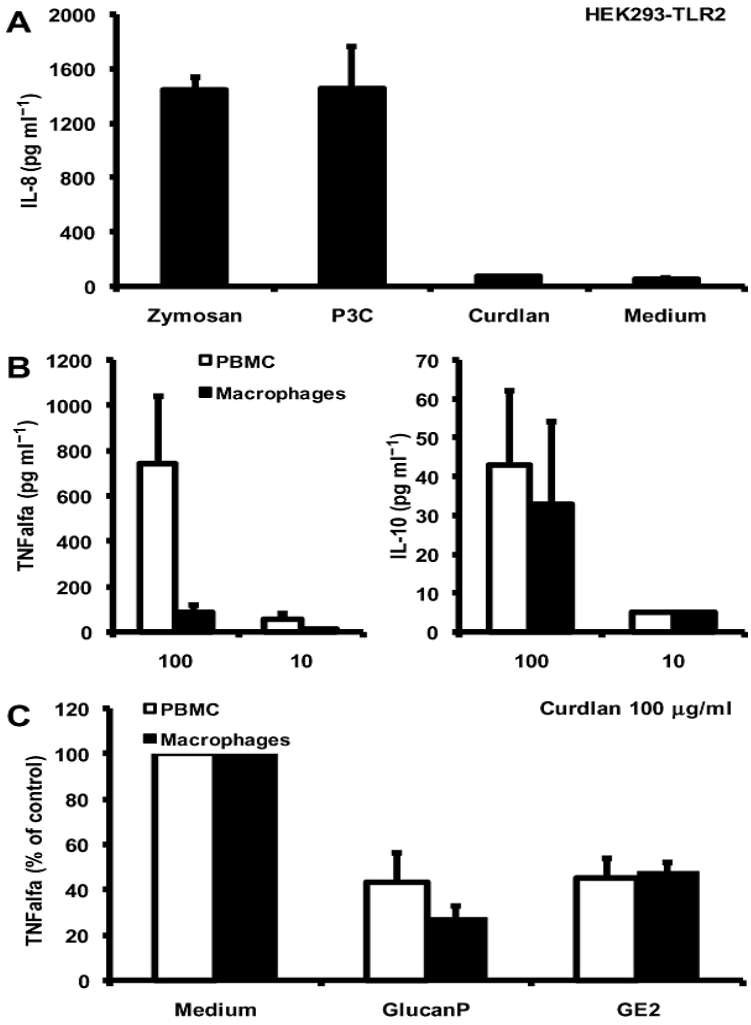

Fig. 3. Curdlan is a specific dectin-1 agonist in human PBMCs and monocyte-derived macrophages. HEK293 cells transiently transfected with human TLR2 were incubated with zymosan (10 µg ml^{-1}), Pam3Cys (1 µg ml^{-1}) or curdlan (100 µg ml^{-1}). After 24 h incubation at 37°C, IL-8 was measured in the supernatant by ELISA. Results are depicted as average of four independent experiments ± SD (A). Human PBMCs and monocyte-derived macrophages of four healthy volunteers were incubated with curdlan at concentrations of 100 or 10 µg ml^{-1}; after 24 h incubation at 37°C, TNFα and IL-10 in the supernatant were measured by ELISA. Data are presented as mean ± SD (B). Human PBMCs and monocyte-derived macrophages of four healthy volunteers were incubated with curdlan (100 µg ml^{-1}) after 1 h pre-incubation with glucanphosphate (100 µg ml^{-1}), anti-dectin-1 antibody GE2 (10 µg ml^{-1}) or matching isotype control (10 µg ml^{-1}). After 24 h incubation at 37°C, TNFα in the supernatant was measured by ELISA. Data are presented as percentage of control (medium stimulated or isotype control) ± SD (C).

However, this does not necessarily mean that similar effects are seen when anti-inflammatory cytokines such as IL-10 are measured, as the regulation of the production of pro- versus anti-inflammatory cytokines is different. In both PBMCs and macrophages medium did not induce any cytokines. The curdlan-induced TNFα production in PBMCs and macrophages could be inhibited with specific dectin-1 inhibitors like glucan phosphate and the dectin-1 antibody GE2 relative to the isotype control [27] (Fig. 3C). Taken together, these data show that curdlan is a specific human dectin-1 agonist without TLR2- or TLR4-stimulating properties.

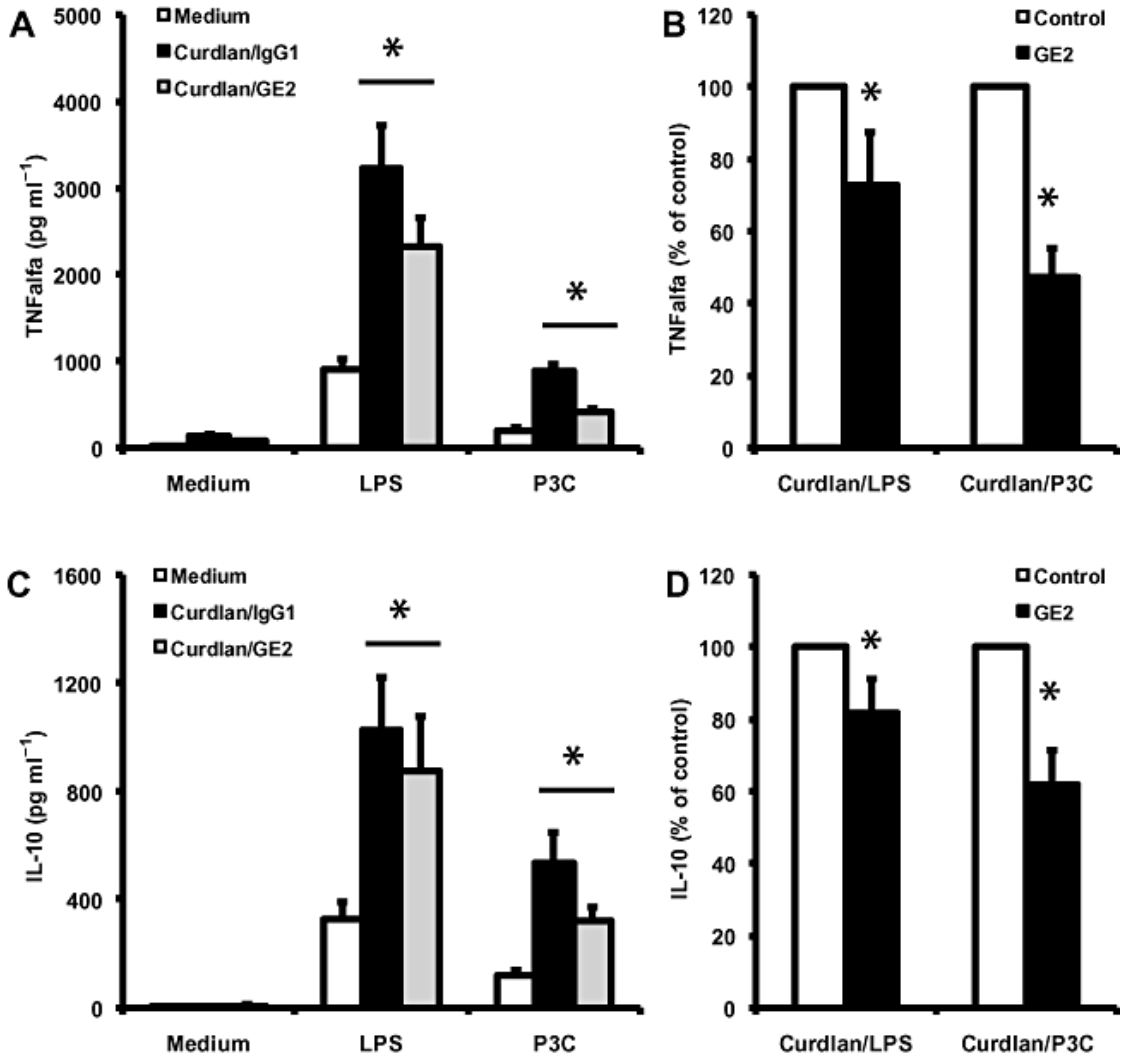

Fig. 4. Synergistic cytokine production after stimulation with curdlan and TLR agonists in human PBMCs is dectin-1-dependent. Human PBMCs of five healthy volunteers were stimulated with LPS (10 ng ml^{-1}) or Pam3Cys (10 µg ml^{-1}) in combination with curdlan (100 µg ml^{-1}). Cells were pre-incubated for 1 h with anti-dectin-1 antibody GE2 (10 µg ml^{-1}) or isotype control (10 µg ml^{-1}). After 24 h incubation at 37°C, TNFα (A and B) and IL-10 (C and D) in the supernatant were measured by ELISA. Data are presented as mean ± SEM and compared by Wilcoxon test (*P < 0.05) (A and C) or as percentage of control ± SD (*P < 0.05) (B and D).

The synergistic cytokine production by PBMCs after stimulation with curdlan and TLR2 or TLR4 agonists is dectin-1-dependent

To investigate the interaction of dectin-1 with TLRs, we stimulated PBMCs with a combination of curdlan with specific TLR agonists. When curdlan was combined with Pam3Cys, a synthetic TLR2 ligand, there was a 2.7 ± 0.3-fold increase in the production of TNFα calculated as [Pam3Cys + curdlan]/([Pam3cys] + [curdlan]) and a 4.2 ± 0.5-fold increase in the production of IL-10 (Fig. 4A and C). The synergistic increase in TNFα production could be inhibited by 53 ± 8% by blocking dectin-1 with the antibody GE2. Under the same experimental conditions, the synergistic IL-10 production was decreased by 38 ± 9% (Fig. 4B and D). When curdlan was combined with the TLR4 ligand LPS, a 3.1 ± 0.2-fold increase in the production of TNFα and a 3.3 ± 0.2-fold increase in the production of IL-10 were detected (Fig. 4A and C). This synergistic production of cytokines could be inhibited with GE2 by 27 ± 15% for TNFα and by 18 ± 9% for IL-10 (Fig. 4B and D).

In summary, these results demonstrate that synergy in cytokine production by PBMCs after stimulation with curdlan in combination with Pam3Cys or LPS is dectin-1-dependent. Interestingly, while synergistic signaling is shown for both dectin-1/TLR2 and dectin-1/TLR4, inhibition of dectin-1 resulted in a more pronounced inhibition of the synergy with TLR2.

Synergy between dectin-1 and TLRs in monocyte-derived macrophages is detected for the pro-inflammatory cytokine TNFα

Because macrophages play a crucial role in the defense against *C. albicans* (Ashman and Papadimitriou, 1995), we further investigated synergistic signaling between dectin-1 and TLR2 or TLR4 in human monocyte-derived macrophages. Similar to the effects in monocytes, the combinations of curdlan with Pam3Cys or LPS exerted robust synergistic effects on cytokine production in human primary macrophages (Fig. 5A and C). The synergy between dectin-1 and TLR2 was most evident for TNFα (13.3 ± 3.9-fold increase), and was inhibited by GE2 by 53 ± 13%. The synergy in IL-10 production (6.5 ± 2.2-fold increase) was not influenced by inhibition of dectin-1 (reduction 22 ± 37%) (Fig. 5B and D). Similarly, synergy between dectin-1 and TLR4 for TNFα secretion (8.8 ± 2.1-fold increase) could be reduced by 72 ± 14% by blocking dectin-1 with GE2 relative to the isotype control. The synergistic production of IL-10 after stimulation with curdlan and LPS was less pronounced (2.1 ± 0.1-fold increase) and not reduced by GE2 (18 ± 11%) (Fig. 5B and D).
Synergistic signaling between dectin-1 and TLR2 or TLR4 in human monocyte-derived macrophages is clearly dectin-1-dependent for the production of the pro-inflammatory cytokine TNFα.

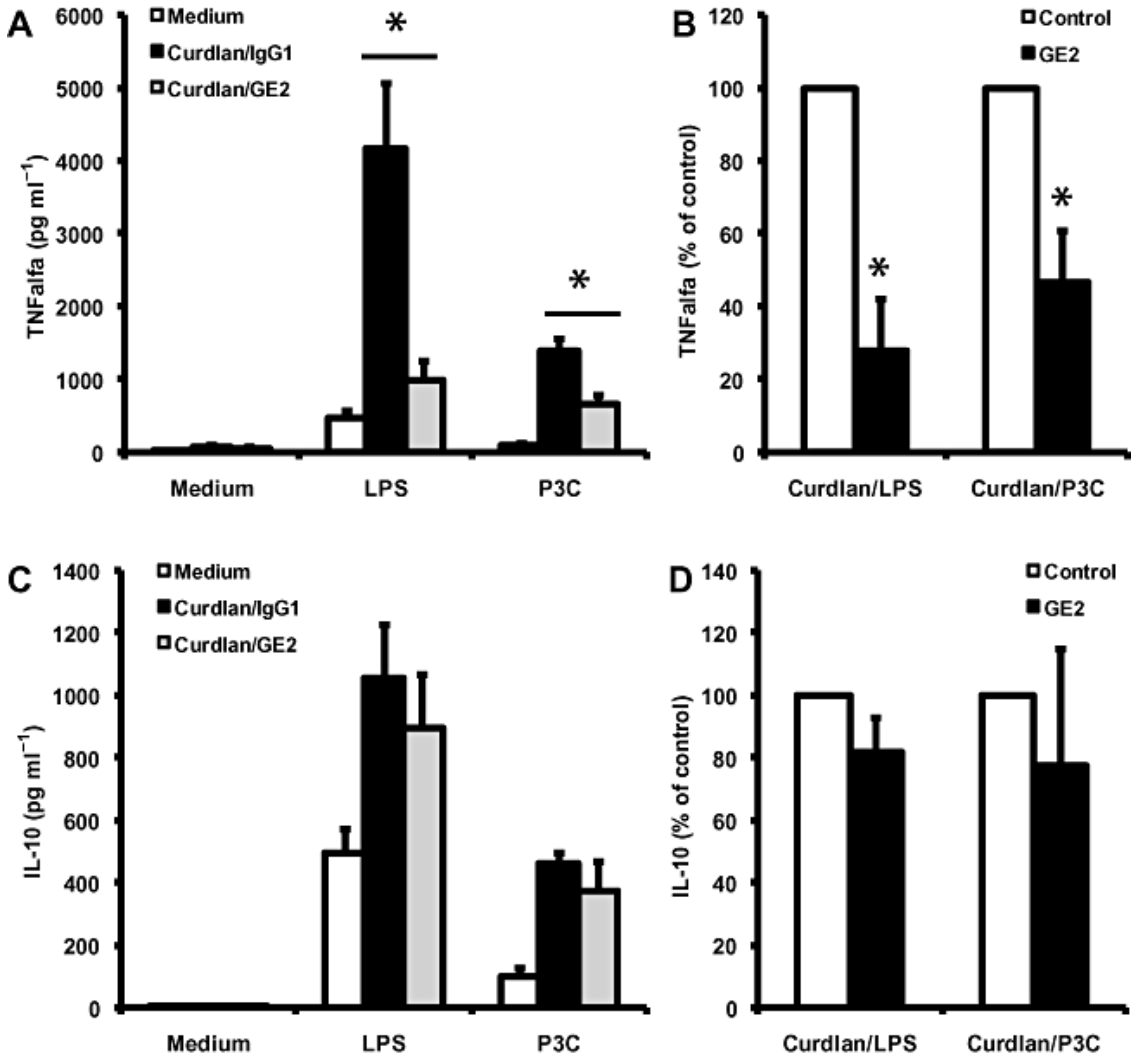

Fig. 5. Synergistic TNFα production after stimulation with curdlan and TLR agonists in human monocyte-derived macrophages is dectin-1-dependent. Human monocyte-derived macrophages of four healthy volunteers were stimulated with LPS (10 ng ml^{-1}) or Pam3Cys (10 µg ml^{-1}) in combination with curdlan (100 µg ml^{-1}). Cells were pre-incubated for 1 h with anti-dectin-1 antibody GE2 (10 µg ml^{-1}) or isotype control (10 µg ml^{-1}). After 24 h incubation at 37°C, TNFα (A and B) and IL-10 (C and D) were measured in the supernatant by ELISA. Data are presented as mean ± SEM and compared by Wilcoxon test (*$P < 0.05$) (A and C) or as percentage of control ± SD (*$P < 0.05$) (B and D).

Discussion

In the present study, we demonstrate that engagement of the β-glucan receptor dectin-1 amplifies cytokine production induced by TLR2 and TLR4 stimulation. Previous studies have reported synergistic effects between dectin-1 and TLR2 in cell lines or mouse macrophages, while we demonstrate synergistic stimulation of primary human monocytes and macrophages by combined stimulation with TLR and CLR ligands. In addition, we report a synergistic interaction between human dectin-1 and TLR4.

Cross-talk between different classes of PRRs is an important mechanism for signal integration to induce specific responses in the innate immune system [28,29]. Receptors can interact with each other at the level of the cell surface and form complexes like TLR4/CD14/MD-2 [30] or TLR2/TLR1 [31] and TLR2/TLR6 [32,33], or at the level of intracellular pathways like Nod2/TLRs [34]. Interactions of dectin-1 with TLR2 have been shown in murine models by using complex particles like zymosan [35,36], which contain both β-glucans and TLR2 ligands (Fig. 3). In addition, we have recently demonstrated synergistic effects of dectin-1 and TLR4 in a murine system [37], and we proposed similar effects for human mononuclear cells. In the present study we extend these observations and demonstrate the strong interaction between dectin-1 and TLR2/TLR4 in both human primary monocytes and macrophages.

The interaction of zymosan with dectin-1 expressed on murine mononuclear cells can have diverse effects. It can trigger TNFα and IL-12 production by cooperative signaling with TLR2. Binding to dectin-1 alone leads to IL-10 and IL-2 secretion [38]. To fully understand these effects, it is pivotal to determine the underlying mechanisms regulating receptor cross-talk. One advantage of using particulate stimuli is that they resemble the cell surface of microorganisms like *C. albicans*. Binding of particulate ligands could approximate receptors to induce synergistic interactions. However, to get more insight into the cross-talk of intracellular pathways, pure receptor ligands are needed. To this end we have used the dectin-1 ligand curdlan, which consists of linear β-1,3-glucan polymers derived from *Alcaligenes faecalis* and does not contain TLR2 or TLR4 ligands (Fig. 3). Our data show that it efficiently triggers dectin-1 in human primary mononuclear cells inducing TNFα release (Fig. 3). When combined with pure TLR2 or TLR4 agonists, it induced dectin-1-dependent synergistic TNFα and IL-10 production (Figs 4 and 5). The synergy for TNFα production was most pronounced in monocyte-derived macrophages. These data show that receptor clustering by binding to a particulate ligand like zymosan is not required for synergistic signaling. Synergy is thus most likely established at the level of signal pathways rather than by interaction of receptors in the cell membrane. Interestingly, synergistic TNFα production was inhibited up to 70% by the dectin-1-blocking antibody GE2 relative to the isotype control in human macrophages. It remains to be elucidated why synergistic IL-10 production was inhibited by GE2 only in PBMCs (Fig. 4) but not in macrophages (Fig. 5). It has been shown that dectin-1 triggering can lead to recruitment of the protein tyrosine kinase Syk to a

phosphorylated ITAM-motif contained in dectin-1 [39]. It should be noted that dectin-1-mediated TNF production is Syk-independent, while IL-10 induction by the β-glucan/dectin−1 interaction is Syk-dependent [40]. Whether Syk dependency of cytokine production is related to difference in the effect of dectin-1 blocking antibody GE2, and whether Syk signaling interferes with TLR2- or TLR4-mediated signaling events in human mononuclear cells, as it has been recently shown in murine cells [41], remain to be determined.

Recently, the intracellular signaling events following dectin-1 triggering have been further elucidated. CARD9 has been shown to control dectin-1-mediated myeloid cell activation, cytokine production and innate antifungal immunity. Card9 couples to Bcl10 and regulates Bcl10-Malt1-mediated NF-κB activation induced by zymosan [42]. Dectin-1 signaling could thus affect TLR-induced intracellular signaling pathways. NF-κB- and ERK-dependent signaling lead to transcription of different cytokines, TNFα in case of NF-κB [43] and IL-10 and IL-2 in case of ERK [44]. Similarly, other ITAM-containing receptors like Fcγ receptors and TREM-1 are thought to synergize with TLRs by augmenting NF-κB- and MAPK-dependent pathways [45,46].

Cross-talk between dectin-1 and TLR2 and TLR4 could play an important role in the human immune response against *C. albicans*. Interestingly, it was shown that *C. albicans* β-glucan is shielded from dectin-1 by outer cell wall components and that yeast budding and cell separation create scars that expose sufficient amounts of β-glucan for dectin-1 triggering on macrophages. During filamentous growth of *C. albicans*, β-glucan is not exposed. Dectin-1 is therefore not triggered, possibly allowing the pathogen to evade an immune response [47]. The synergy between dectin-1 and TLR2 and TLR4 for the induction of cytokines is interesting in perspective of *Candida* infections, because each layer of the *C. albicans* cell wall can trigger specific receptors. Cross-talk between these receptors will lead to an optimal immune response of the host. The immunomodulatory properties of β-glucans in human mononuclear cells could go further than host defense against *Candida spp*. Considering our findings that stimulation with a pure dectin-1 ligand in combination with pure TLR ligands leads to synergic cytokine production in human mononuclear cells, one could imagine using pure dectin-1 ligands as adjuvants to increase immune responses. Insights gained into cross-talk between TLRs and dectin-1 will increase our understanding of how the immune responses against *C. albicans* are orchestrated and may help to improve current therapeutic approaches.

Materials and methods

Reagents

Synthetic Pam3Cys was purchased from EMC Microcollections. LPS (*E. coli* serotype 055:B5) was purchased from Sigma and an extra purification step was performed as described previously [48]. Purified LPS was tested in TLR4−/− mice for the presence of contaminants and did not have any TLR4-independent activity [49]. Zymosan (Sigma), prepared from the *Saccharomyces cerevisiae* cell wall, consists of protein–carbohydrate complexes and contains high amounts of β-glucans. Curdlan (WAKO) is a dectin-1 ligand consisting of linear β-1,3-glucan polymers derived from *Alcaligenes faecalis* [50-52]. The inhibiting antibody against dectin-1, GE2, was a kind gift of Prof Gordon Brown (University of Cape Town, Cape Town, South Africa). A matching mIgG1 isotype control was purchased from R&D systems. Endotoxin-free water-soluble glucan phosphate was prepared from particulate β-glucan as previously described [53]. The mouse anti-human monoclonal anti-TLR4 HTA125 antibody (aTLR4) was a kind gift of Dr Kensuke Miyake (Saga Medical School, Saga, Japan) and a matching mIgG2a isotype was purchased from R&D systems. The mouse anti-human monoclonal anti-TLR2 antibody (aTLR2, isotype IgG2a) was kindly provided by Dr Douglas Golenbock (University of Massachusetts, Boston, USA).

Isolation of PBMCs and stimulation of cytokine production

After obtaining informed consent, venous blood was drawn from the cubital vein of healthy volunteers into three 10 ml EDTA tubes (Monoject). The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and suspended in culture medium (RPMI 1640, Cambrex) supplemented with gentamicin 10 μg ml−1, l-glutamine 10 mM and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5×10^6 cells ml−1. A total of 5×10^5 MNCs in a 100 μl volume were added to round-bottom 96-wells plates (Greiner) and incubated with either 100 μl of culture medium (negative control), or the various stimuli: LPS (1 ng ml^{-1}), Pam3Cys (1 μg ml^{-1}) and curdlan (100 μg ml^{-1}) or combinations of curdlan/Pam3Cys and curdlan/LPS. In parallel experiments, dectin-1 was inhibited by adding 10 μg ml^{-1} GE2 or matched isotype control 30 min before adding the stimuli. After 24 h, the supernatants were collected and stored at −70°C until assayed.

Culture of human monocyte-derived macrophages

To culture human macrophages, 5×10^5 PBMCs were plated in flat-bottom 96-wells plates. After 4 h culture at 37°C, they were washed three times with RPMI1640. The adhered monocytes were cultured for at least 6 days in RPMI1640 with 10% heat-inactivated pooled human serum, until they showed macrophage-like morphology and expressed characteristic surface markers analyzed by flow cytometry. After washing three times macrophages were stimulated according to the same protocol as PBMCs.

Cytokine measurements

Human TNFα concentrations were determined by a specific ELISA using four antibodies as described by [54]. In short, the procedure started with treating microtitre plates with a duck-anti-chicken IgY coating antibody solution (overnight at 4°C). Afterwards, the plates were blocked with the blocking buffer (300 µl per well, 2 h at 37°C). The next step was incubation with chicken anti-TNFα capture antibodies (2 h at 37°C). The incubation with the standards, unknowns and reference samples took place overnight at 4°C. The incubation with trapping antibody (rabbit anti-TNFα), as well as the subsequently incubation with detection antibody (HRP-labeled goat anti-rabbit antibody) were performed over 2 h at ambient temperature. The incubation with substrate solution was performed in darkness for 60 min at ambient temperature. When the color reaction was completed, 1 M H2SO4 per well was added to stop the reaction. The absorbance values were measured at 492 nm within 30 min. IL-10 and IL-8 were measured by commercial ELISA kits (Pelikine Compact, Sanquin), according to the instructions of the manufacturer.

Transfection of HEK293 cells

HEK293 cells were cultured in complete DMEM (Life Technologies, Gibco BRL)/10% FCS. Transfections were done with lipofectamine according to the manufacturer's instructions (Invitrogen). Human TLR2 constructs were a kind gift of Dr Douglas Golenbock (University of Massachusetts, Boston, USA).

Flow cytometry

Polyclonal goat anti-human dectin-1 antibodies (R&D Systems) were used for flow cytometry (FACS) analysis of dectin-1 expression. Donkey anti-goat Alexa647-conjugated antibody (Molecular Probes) was used to detect anti-human dectin-1 antibody in FACS studies. Murine monoclonal anti-MR and anti-CD14 antibodies (clone 19.2, BD, and RMO52, Beckman Coulter respectively) and PE-conjugated secondary antibodies (BD) were used to label monocytes and macrophages. FACS analyses were performed with a FACScalibur (BD). FACS data were analyzed with WinMDI (http://facs.scripps.edu/software.html) and CellQuest (BD).

Statistical analysis

All experiments were performed in duplicate with cells obtained from at least four healthy volunteers. Synergy was expressed as ratio of cytokine response of ligand in combination with curdlan divided by the sum of cytokine responses obtained with each ligand alone. Differences in cytokine production after inhibition of dectin-1 were analyzed by Wilcoxon tests. For all other comparisons the Student's t-test was used. The level of significance between groups was set at $P < 0.05$. Data are given as mean ± SEM.

Reference List

1 Pfaller,M.A. and Diekema,D.J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20, 133-163

2 Akira,S. *et al.* (2006) Pathogen recognition and innate immunity. *Cell* 124, 783-801

3 Takeda,K. *et al.* (2003) Toll-like receptors. *Annu. Rev. Immunol.* 21, 335-376

4 Figdor,C.G. *et al.* (2002) C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol.* 2, 77-84

5 Cambi,A. *et al.* (2005) How C-type lectins detect pathogens. *Cell Microbiol.* 7, 481-488

6 Cambi,A. *et al.* (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *Eur. J. Immunol.* 33, 532-538

7 Netea,M.G. *et al.* (2006) Recognition of fungal pathogens by toll-like receptors. *Curr. Pharm. Des* 12, 4195-4201

8 Willment,J.A. and Brown,G.D. (2008) C-type lectin receptors in antifungal immunity. *Trends Microbiol.* 16, 27-32

9 Jouault,T. *et al.* (2003) Candida albicans phospholipomannan is sensed through toll-like receptors. *J. Infect. Dis.* 188, 165-172

10 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

11 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

12 Brown,G.D. and Gordon,S. (2001) Immune recognition. A new receptor for beta-glucans. *Nature* 413, 36-37

13 Willment,J.A. *et al.* (2001) Characterization of the human beta -glucan receptor and its alternatively spliced isoforms. *J. Biol. Chem.* 276, 43818-43823

14 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

15 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

16 Netea,M.G. *et al.* (2002) The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J. Infect. Dis.* 185, 1483-1489

17 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

18 Netea,M.G. *et al.* (2004) Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* 172, 3712-3718

19 Netea,M.G. *et al.* (2004) Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* 172, 3712-3718

20 Sutmuller,R.P. *et al.* (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest* 116, 485-494

21 Netea,M.G. *et al.* (2002) The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J. Infect. Dis.* 185, 1483-1489

22 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

23 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

24 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

25 Underhill,D.M. (2007) Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. *Immunol. Rev.* 219, 75-87

26 Wewers,M.D. and Herzyk,D.J. (1989) Alveolar macrophages differ from blood monocytes in human IL-1 beta release. Quantitation by enzyme-linked immunoassay. *J. Immunol.* 143, 1635-1641

27 Willment,J.A. *et al.* (2005) The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells. *Eur. J. Immunol.* 35, 1539-1547

28 Lee,M.S. and Kim,Y.J. (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu. Rev. Biochem.* 76, 447-480

29 Underhill,D.M. (2007) Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. *Immunol. Rev.* 219, 75-87

30 Akashi,S. *et al.* (2000) Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. *Biochem. Biophys. Res. Commun.* 268, 172-177

31 Jin,M.S. *et al.* (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130, 1071-1082

32 West,A.P. *et al.* (2006) Recognition and signaling by toll-like receptors. *Annu. Rev. Cell Dev. Biol.* 22, 409-437

33 Gay,N.J. and Gangloff,M. (2007) Structure and function of Toll receptors and their ligands. *Annu. Rev. Biochem.* 76, 141-165

34 Netea,M.G. *et al.* (2005) Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J. Immunol.* 174, 6518-6523

35 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

36 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

37 Dennehy,K.M. *et al.* (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* 38, 500-506

38 Brown,G.D. (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 6, 33-43

39 Rogers,N.C. *et al.* (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity.* 22, 507-517

40 Gow,N.A. *et al.* (2007) Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* 196, 1565-1571

41 Dennehy,K.M. *et al.* (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* 38, 500-506

42 Gross,O. *et al.* (2006) Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442, 651-656

43 Collart,M.A. *et al.* (1990) Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol. Cell Biol.* 10, 1498-1506

44 Slack,E.C. *et al.* (2007) Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan. *Eur. J. Immunol.* 37, 1600-1612

45 Radsak,M.P. *et al.* (2004) Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J. Immunol.* 172, 4956-4963

46 Hu,X. *et al.* (2007) Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *J. Leukoc. Biol.* 82, 237-243

47 Gantner,B.N. *et al.* (2005) Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *EMBO J.* 24, 1277-1286

48 Hirschfeld,M. *et al.* (2000) Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* 165, 618-622

49 Sutmuller,R.P. *et al.* (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest* 116, 485-494

50 Palma,A.S. *et al.* (2006) Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. *J. Biol. Chem.* 281, 5771-5779

51 Yoshitomi,H. *et al.* (2005) A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201, 949-960

52 Meyer-Wentrup,F. *et al.* (2007) Dectin-1 interaction with tetraspanin CD37 inhibits IL-6 production. *J. Immunol.* 178, 154-162

53 Williams,D.L. *et al.* (1991) A method for the solubilization of a (1----3)-beta-D-glucan isolated from Saccharomyces cerevisiae. *Carbohydr. Res.* 219, 203-213

54 Grebenchtchikov,N. *et al.* (2005) Development of a sensitive ELISA for the quantification of human tumour necrosis factor-alpha using 4 polyclonal antibodies. *Eur. Cytokine Netw.* 16, 215-222

Chapter 3

Syk kinase is required for collaborative cytokine production induced through dectin-1 and Toll-like receptors

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Abstract

Recognition of microbial components by germ-line encoded pattern recognition receptors (PRR) initiates immune responses to infectious agents. We and others have proposed that pairs or sets of PRR mediate host immunity. One such pair comprises the fungal β-glucan receptor, Dectin-1, which collaborates through an undefined mechanism with Toll-like receptor 2 (TLR2) to induce optimal cytokine responses in macrophages. We show here that Dectin-1 signaling through the spleen tyrosine kinase (Syk) pathway is required for this collaboration, which can also occur with TLR4, 5, 7 and 9. Deficiency of either Syk or the TLR adaptor MyD88 abolished collaborative responses, which include TNF, MIP-1 and MIP-2 production, and which are comparable to the previously described synergy between TLR2 and TLR4. Collaboration of the Syk and TLR/MyD88 pathways results in sustained degradation of the inhibitor of κB (IκB), enhancing NF-κB nuclear translocation. These findings establish the first example of Syk- and MyD88-coupled PRR collaboration, further supporting the concept that paired receptors collaborate to control infectious agents.

Introduction

Originally identified in Drosophila, the Toll-like receptors (TLR) consist of a family of at least 11 proteins, which recognize a diverse, but receptor-specific range of microbial structures. Ligand recognition leads to TLR homo- or heterodimerization and the initiation of specific signaling cascades mediated by the intracellular adaptors MyD88 and TRIF [1]. This leads to the activation of transcription factors, including NF-κB, inducing TLR-specific patterns of gene expression. However, the specificity of these responses is incompletely understood and is thought to stem, at least in part, from the association with particular intracellular adaptors, heterodimerization, synergy between selected TLR, and the contribution of other non-TLR pattern recognition receptor (PRR) that are associated with the recognition of specific microbes[1-5]. Recently, it has been proposed that complex sets of PRR collaborate to mediate host immune responses to intact microbes [2]. For fungi, one such set comprises the β-glucan receptor Dectin-1 and TLR2 [5,6].

Dectin-1 is essential for the innate response to fungal pathogens [7]. *In vitro* studies have shown that recognition of fungal β-1,3-glucan by Dectin-1 can induce phagocytosis, phospholipase A2, COX2, the respiratory burst and the production of numerous cytokines and chemokines, including TNF, MIP-2, IL-12, IL-2, IL-10, IL-6 and IL-23 [5]. Signaling from Dectin-1 is mediated through novel pathways, including an unusual interaction with spleen tyrosine kinase (Syk) which triggers downstream signaling through CARD9 [5,8]. While the activation of Syk is sufficient for the induction of the respiratory burst in macrophages and IL-10 and IL-23 in dendritic cells [9-11], the role of this kinase in the induction of the other cytokines and chemokines, if any, is unclear. Furthermore, in macrophages, some of Dectin-1-mediated responses, such as phagocytosis, are Syk-independent, while others, such as the induction of TNF, require collaborative recognition of another undefined fungal component by TLR2, and signaling through the MyD88 pathway [5,12-14].

How Dectin-1 and TLR2 collaborate to induce proinflammatory cytokines and chemokines is not understood. We show here that this receptor collaboration requires the Syk kinase pathway, utilized by Dectin-1, and that this pathway can collaborate with many other TLR to induce optimal cytokine and chemokine responses. Collaboration of these pathways results in sustained IκB degradation and enhanced NF-κB nuclear translocation. These results demonstrate the importance of receptor collaboration during infection as opposed to receptors functioning in isolation.

Results and discussion

Collaboration of dectin-1 and TLR2 using specific ligands

Dectin-1 and TLR2 are known to collaborate to induce cytokine production in response to fungal particles [12,13]. In order to determine the nature of this collaboration, we stimulated thioglycollate-elicited macrophages with highly purified ligands specific for each receptor [15]. Stimulation with purified β-glucan failed to induce TNF production, even at high doses (Fig. 1A). Given the surprising lack of response to the purified β-glucan, we measured whether Syk kinase was being activated by this ligand, as an indicator of Dectin-1 activation [9] (Fig. 1B and C). Both Syk, and its substrate SLP-76, were phosphorylated following β-glucan stimulation, indicating that engagement of Dectin-1 and activation of the Syk pathway is not sufficient to induce TNF production in macrophages.

To explore collaborative signaling between Dectin-1 and TLR2, we added sub-stimulatory doses of the TLR2-specific ligand, Pam3CSK4, alone or in combination with β-glucan (Fig. 1A). While stimulation with the low dose of TLR2 ligand induced low levels TNF, a combination of the two ligands induced high levels of this cytokine, suggesting that the Dectin-1 and TLR2 ligands acted in a synergistic fashion for inflammatory cytokine production. Similar results were also obtained with curdlan, another particulate β-glucan used as a Dectin-1 ligand (data not shown). Synergistic induction of TNF was most evident at lower concentrations of Pam3CSK4 (Fig. 1D), and was not observed in Dectin-1-/- macrophages (Fig. 1E). Chemokines such as MIP-1 and MIP-2 were also efficiently induced by co-ligation but not by β-glucan alone, indicating that synergistic stimulation of TLR2 and Dectin-1 can induce a variety of macrophage responses (Fig. 1F and G). As TNF and MIP-1 production after TLR2 stimulation never reach the levels obtained after co-stimulation, regardless of ligand concentration (Fig. 1D and F), these data suggest that such responses are qualitatively different and not simply quantitative shifts in the dose-response curve.

Dectin-1 and TLR2 collaboration requires MyD88 and Syk kinase-signaling pathways

Collaboration between Dectin-1 and TLR2 in response to fungal particles could occur in two ways. First, Dectin-1 could capture fungal particles and present ligands to TLR2 at the cell surface or in the phagosome, similar to that proposed for CD36 [3]. Since Syk is not required for binding and phagocytosis of fungal particles by macrophages, such collaboration would be dependent on MyD88 and not Syk, as previously suggested [5,14]. Alternatively, receptor collaboration could require signaling through both receptors and signal integration. In this scenario, collaboration would require both MyD88 and possibly Syk. To test these possibilities, we examined thioglycollate-elicited macrophages derived from wild-type, MyD88-/- and Syk-/- chimeric mice (Fig. 2A and B).

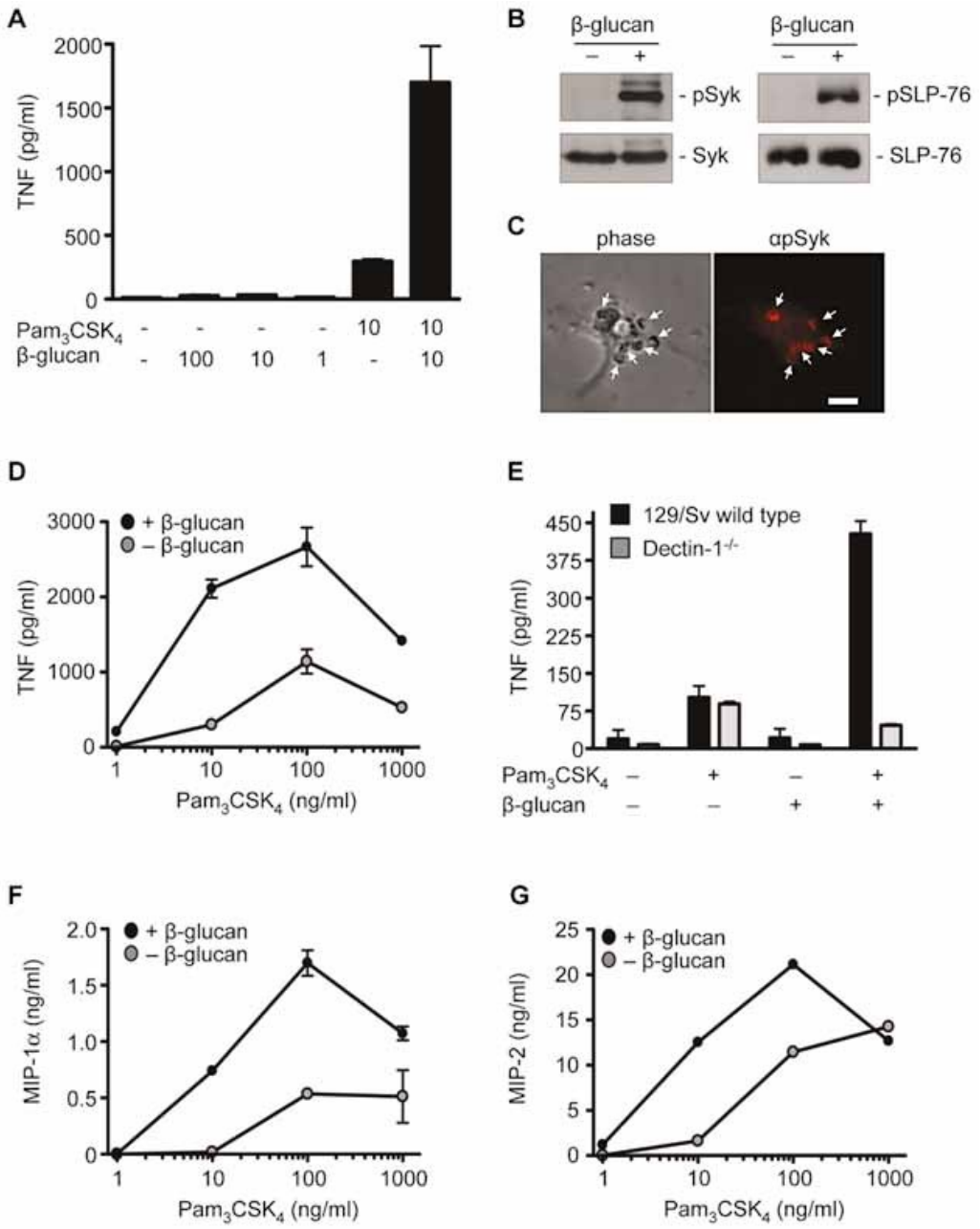

Figure 1. Collaborative induction of pro-inflammatory cytokines through Dectin-1 and TLR2 using specific ligands. (A) Production of TNF by C57BL/6 macrophages stimulated with 100, 10 or 1 µg/mL particulate β-glucan, with or without 10 ng/mL Pam3CSK4. (B) Western blots showing phosphorylation of Syk, and its substrate SLP-76, upon stimulation of RAW macrophages with 10 µg/mL β-glucan. Data shown are representative of two independent experiments. (C) Activation of Syk in 129Sv macrophages by β-glucan (arrows), as determined by fluorescence microscopy. Left panel, phase image; right panel phospho-Syk image. Scale bar indicates 10 m. (D) Collaborative production of TNF by C57BL/6 macrophages stimulated with 10 µg/mL particulate β-glucan occurs over a range of Pam3CSK4 concentrations. (E) Collaborative TNF production by 129/Sv macrophages stimulated with 10 µg/mL particulate β-glucan and 10 ng/mL Pam3CSK4 is dependent on Dectin-1 expression. Collaborative MIP-1 (F) and MIP-2 (G) production from C57BL/6 macrophages stimulated with 10 µg/mL particulate β-glucan occurs over a range of Pam3CSK4 concentrations. Data shown are mean ± SD and are representative of at least two independent experiments.

In wild-type macrophages from C57BL/6 and BALB/C mice, prominent TNF production was induced by a combination of the two ligands, as before. In contrast, this response was absent in MyD88-/- macrophages (Fig. 2A). However, responses were similarly defective in Syk-/- macrophages, in which the level of TNF production was comparable to that obtained by TLR2 ligation alone (Fig. 2B). These results indicate that efficient induction of specific cytokine responses in macrophages requires integration of the Dectin-1/Syk and TLR/MyD88 signaling pathways, and can thus be defined as synergistic or collaborative responses.

Receptor collaboration sustains degradation of IκB and enhances nuclear translocation of NF-κB

Both the TLR/MyD88 and Syk pathways couple to IκB and NF-κB activation, ultimately leading to cytokine and chemokine induction [1,8]. However, signaling through the MyD88 pathway alone induces only transient IκB degradation, and subsequent transient NF-κB nuclear translocation in mouse embryonic fibroblasts [16]. Similarly, activation of either the TLR2/MyD88 or Dectin-1/Syk kinase pathways alone induced transient IκB degradation in a macrophage cell line, with IκB protein levels largely recovering after 120 min (Fig. 2C and D). In contrast, co-stimulation induced sustained IκB degradation, which correlated with enhanced nuclear localization of NF-κB c-Rel (Fig. 2C and D). In order to verify these findings in primary cells, we measured NF-κB c-Rel nuclear translocation microscopically over time in thioglycollate-elicited peritoneal macrophages (Fig. 2E and F). At early time points following stimulation, the percentages of cells with nuclear c-Rel were significantly greater after co-stimulation, than after stimulation of either receptor alone (Fig. 2F). However, at later time points there was no significant difference in nuclear c-Rel translocation between the co-stimulated cells and the cells stimulated with TLR ligand alone. Although we have not defined the point of signal integration, we show that while ligation of either receptor can induce activation of NF-κB, collaboration of the signals induced by these receptors results in sustained IκB degradation and NF-κB nuclear localization at earlier time points, which is likely to contribute, at least in part, to the enhanced cytokine responses.

The Syk kinase pathway collaborates with TLR2, 4, 5, 7 or 9

Given that most TLR signal through MyD88, we examined whether the Syk kinase pathway can collaborate with other MyD88-coupled TLR. Stimulation of human peripheral blood mononuclear cells with suboptimal doses of TLR1/2, TLR4 and TLR5 ligands induced low levels of TNF that were greatly enhanced by activation of the Syk pathway with β-glucan (Fig. 3A, B and C). Similarly, co-ligation of TLR7 or TLR9 with Dectin-1 induced collaborative TNF responses in thioglycollate-elicited macrophages (Fig. 3D). Signaling through TLR/MyD88 is thus a general pathway for collaboration with the Syk kinase pathway.

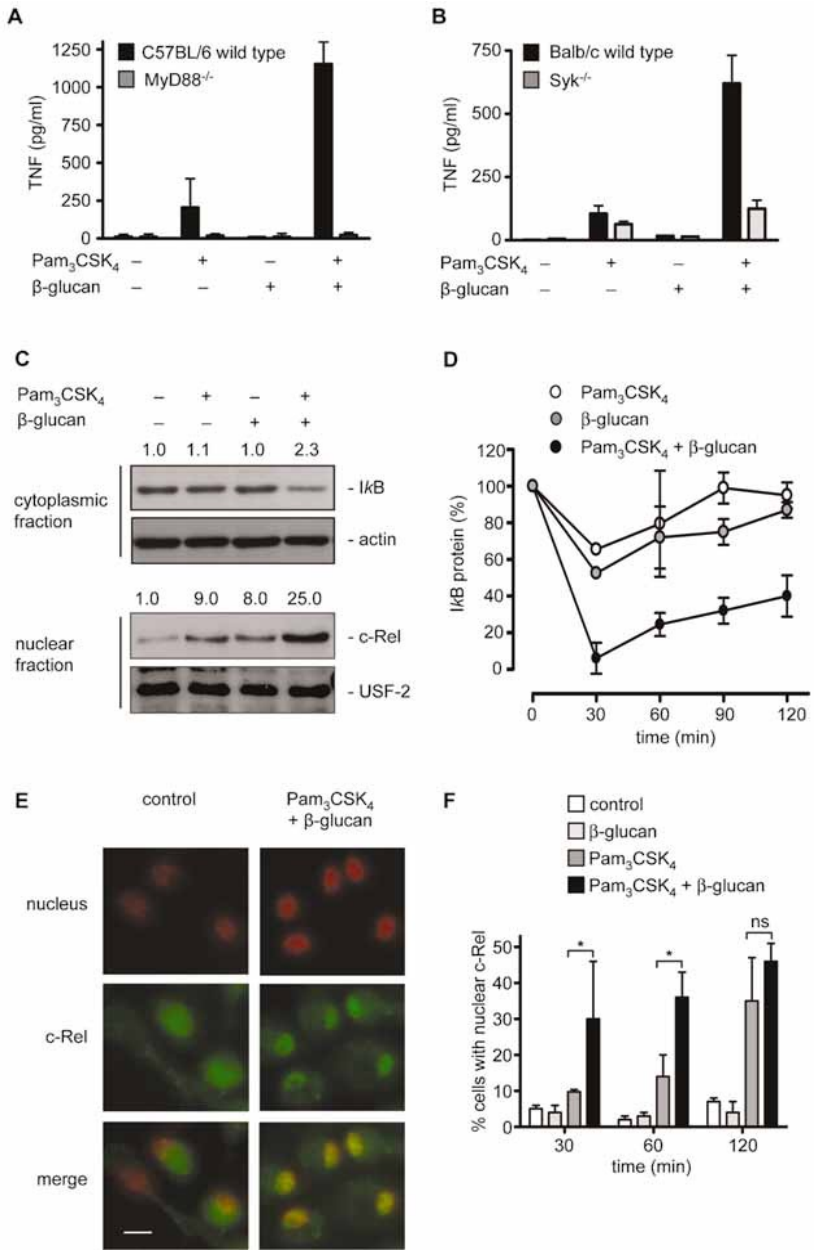

Figure 2. Collaboration of Syk kinase and TLR/MyD88 pathways sustain IκB degradation and enhance NF-κB nuclear translocation. TNF production from C57BL/6 wild-type and MyD88-/- (A) or BALB/C wild-type and Syk-/- (B) macrophages stimulated with 10 μg/mL particulate β-glucan and 10 ng/mL Pam3CSK4, as indicated. (C) RAW macrophages stimulated with 10 ng/mL Pam3CSK4 and 10 μg/mL particulate -glucan. Top: IκB degradation after 2 h was assayed by Western blotting. Bottom: localization of NF-κB c-Rel in nuclear lysates. Numbers above each lane show fold decrease (IκB) or increase (cRel) of the relative band intensities of IκB and c-Rel, with actin and USF-2 as loading controls, versus unstimulated control. (D) IκB degradation in RAW macrophages was assayed after the indicated times. Data shown are mean ± SD and are representative of two independent experiments. (E) Nuclear translocation of c-Rel (green) following co-stimulation of C57BL/6 macrophages with 10 ng/mL Pam3CSK4 and 10 μg/mL particulate β-glucan for 1 h. Nuclei were stained with Hoechst 33258 (red). Scale bar represents 10 m. (F) Nuclear translocation of c-Rel was quantified microscopically over time in C57BL/6 macrophages stimulated with 10 ng/mL Pam3CSK4 followed by 10 μg/mL particulate β-glucan. Data shown are mean ± SD and are representative of two independent experiments.

Receptor synergy has previously been described in innate immunity, notably between TLR that signal through MyD88 and TRIF adaptors respectively [2,17-19]. Collaborative TNF responses after co-ligation of Dectin-1 with either TLR2 or TLR4 in thioglycollate-elicited macrophages were comparable to the previously described synergistic responses between these two TLR (Fig. 3E). As β-glucans have long been known to stimulate anti-infective immunity [5], our results suggest that the activity of these carbohydrates stems from their ability to trigger collaborative responses between Dectin-1 and the various TLR. Similar to specific TLR ligand combinations, the collaborative effects of Dectin-1 and TLR may provide an alternative approach for the development of novel adjuvants.

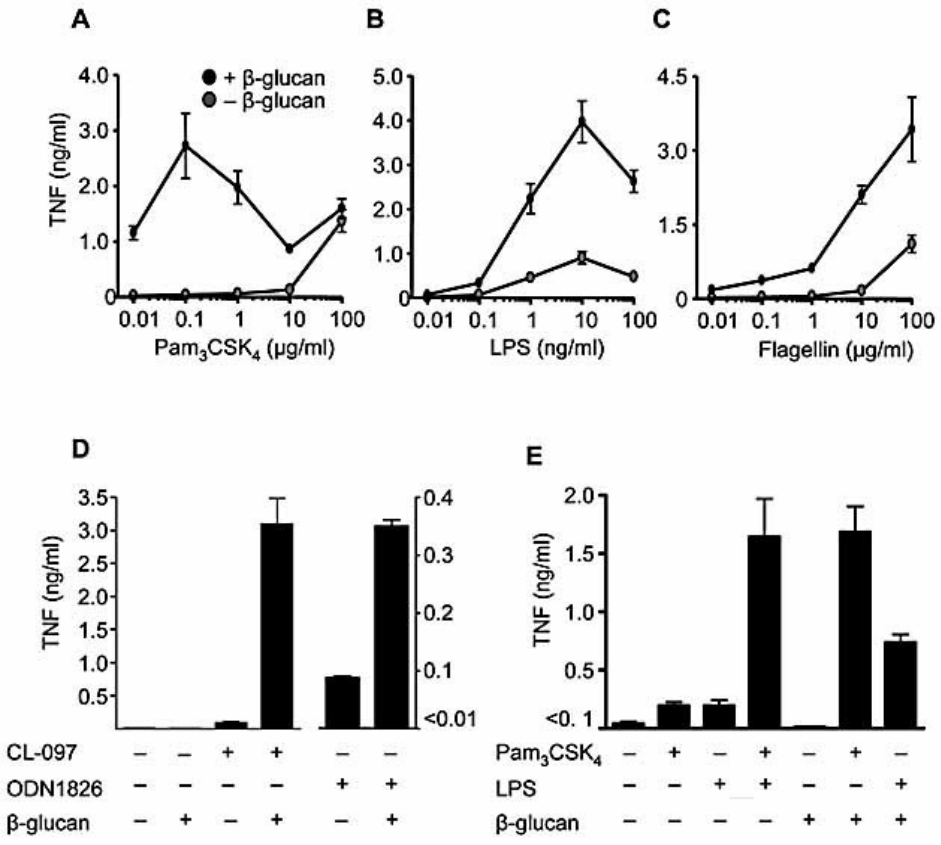

Figure 3. Collaborative responses occur with multiple TLR and are comparable to synergistic responses induced through TLR2 and TLR4. Production of TNF by human peripheral blood mononuclear cells stimulated with or without 10 μg/mL particulate β-glucan and the indicated concentrations of Pam3CSK4 (A), LPS (B) or flagellin (C). Data shown are mean ± SEM of pooled data from five independent donors. (D) Collaborative TNF responses from C57BL/6 macrophages stimulated with TLR7 (0.2 μg/mL CL-097) or TLR9 (1 μg/mL ODN1826) ligands and particulate β-glucan, as indicated. Data shown are the mean ± SD of one representative experiment of three. (E) Co-stimulation of C56BL/6 macrophages with 10 μg/mL particulate β-glucan and 10 ng/mL Pam3CSK4 or 1 ng/mL LPS induces TNF production that is comparable to the synergistic response following TLR2 and TLR4 co-ligation. Shown are the mean ± SEM pooled of data pooled from three independent experiments.

Concluding remarks

In this study, we have used defined ligands to explore the nature of the collaborative response induced by the β-glucan receptor, Dectin-1, and TLR2. We show here that the Syk kinase pathway utilized by Dectin-1 is required for this collaboration, and demonstrate that this pathway can also collaborate with TLR4, 5, 7 and 9. The collaborative responses between Dectin-1 and the TLR may be particularly relevant for intact microbes bearing the appropriate combination of ligands. Indeed, the susceptibility to fungal infection of mice deficient in Dectin-1 [7], or the downstream signaling pathway [8], despite normal responses to specific TLR ligands, indicates that the defect in these animals is due to the lack of collaborative signaling from the Dectin-1 pathway. Receptor collaboration may also explain the previously observed synergy between mannans, which may be TLR ligands, and β-glucan fungal cell wall components in the response to these organisms [7,20]. These results thus highlight the importance of receptor collaboration during infection as opposed to receptors functioning in isolation, and further support the concept that pairs or sets of receptors collaborate to control infectious agents.

While Syk has been implicated in the modulation of TLR responses in other systems [10,21-24], the interaction between Dectin-1 and Syk occurs through a novel mechanism [9], which is likely to be representative of other receptors involved in pathogen recognition such as the HIV-1 receptor CLEC-2 and CD66d, a PRR for Neisseria gonorrhoeae and Haemophilus influenzae [25-28]. Recently, it has also been proposed that the adaptor CARD9, which is downstream of Syk in myeloid cells, plays a role in TLR signaling [29]. We propose an alternative explanation in which Syk-and CARD9-coupled PRR collaborate with TLR. Stimulation of TLR9 with CpG, for example, induces IL-12p70 production that is dependent on Syk [10], suggesting that an undefined Syk- and CARD9-coupled PRR collaborates with TLR9 in this system. There are therefore likely to be many more examples of Syk-coupled PRR that collaborate with TLR to control infectious agents.

Materials and methods

Reagents and mice

All TLR ligands were from InvivoGen (San Diego, CA), and the production of highly purified particulate β-glucan and soluble β-glucan (glucan phosphate) have been described elsewhere [15,30]. BALB/c, C57BL/6 and C57BL/6 MyD88-/-, 129/Sv and 129/Sv Dectin-1-/- [7] mice were obtained from the animal unit of the University of Cape Town. BALB/c Syk-/- chimeric mice were generated by the transfer of Syk-/- fetal liver cells into irradiated BALB/C recipients as described [9]. All procedures were approved by the University of Cape Town animal ethics committee.

Cell stimulation

Murine thioglycollate-elicited macrophages were plated at 10^6 cells/mL in RPMI medium containing 10% FCS (Gibco) and incubated overnight. Medium was replaced, and cells were stimulated with 10 μg/mL particulate β-glucan or 10 ng/mL Pam3CSK4, unless otherwise indicated, for 3 h. Human peripheral monocytes were prepared as described [20,31] and stimulated with 10 μg/mL particulate β-glucan and relevant TLR ligands for 20 h. Cytokine secretion was assayed by ELISA using kits from Becton Dickinson (Mountain View, CA), R&D Systems (Abbingdon, UK) or KOMA Biotechnology (Korea). Data were analyzed using the Student's t-test.

Syk and SLP-76 phosphorylation

RAW 264.7 cells expressing Dectin-1 [12] (10^7cells in 100 L HBSS) were stimulated with 10 μg/mL soluble β-glucan (glucan phosphate) for 1 min at 37°C before addition of lysis buffer (1% NP40, 25 mM Tris pH 8, 10 mM EDTA, 140 mM NaCl, 5 mM NaF, 1 mM Na3VO4, 5 mM iodoacetamide) containing protease inhibitors (Roche). Lysate supernatants were incubated for 2 h with 30 L streptavidin-agarose beads (Fluka) precoated with 25 M biotinylated Dectin-1 phosphopeptide to precipitate Syk [9] or 30 L protein G-Sepharose beads (Amersham, UK) precoated with 5 g SLP-76 mAb. Beads were washed with lysis buffer, boiled in 50 L SDS-PAGE sample buffer, and samples were Western-blotted and probed with phosphotyrosine mAb 4G10 (Becton Dickinson) and antibodies to Syk or SLP-76 (Santa Cruz Biotechnology, CA). For phospho-Syk staining, 129Sv thioglycollate-elicited macrophages were stimulated with 10 μg/mL particulate β-glucan for 6 min, fixed with paraformaldehyde, blocked and permeabilized with Triton X-100. Cells were stained with anti-phosphoSyk (CellSignalling) followed by donkey anti-rabbit Cy3 (Jackson Immunoresearch) and analyzed by fluorescent microscopy.

IκB degradation and NF-κB nuclear localization

RAW 264.7 cells expressing Dectin-1 plated at 2×10^5 cells/mL were stimulated with 10 μg/mL particulate β-glucan and 10 ng/mL Pam3CSK4 for 2 h, or the indicated times, before lysis and Western blotting of whole-cell lysates with antibodies to IκB or actin (CellSignaling, MA) as a loading control. Nuclear lysates were prepared after 20 h stimulation as described

[32], followed by Western blotting with antibodies to NF-κB c-Rel or USF-2 (Santa Cruz Biotechnology) as a loading control. Band intensities were quantified using NIHImage software.

NF-κB nuclear translocation

C57BL/6 thioglycollate-elicited macrophages plated at 2×10^5 cells/mL were stimulated with 10 ng/mL Pam3CSK4 for 90 min followed by 10 μg/mL particulate β-glucan for 60 min, fixed with paraformaldehyde, blocked and permeabilized with Triton X-100. Cells were stained with Hoechst and NF-κB c-Rel antibody, followed by anti-rabbit IgG-Cy6 (Jackson laboratories, USA) and analyzed by fluorescent microscopy. Three independent fields containing >100 cells were counted for each stimulation type, and data were analyzed using the Student's t-test.

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Reference List

1 O'Neill,L.A. (2006) How Toll-like receptors signal: what we know and what we don't know. *Curr. Opin. Immunol.* 18, 3-9

2 Trinchieri,G. and Sher,A. (2007) Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7, 179-190

3 Hoebe,K. *et al.* (2005) CD36 is a sensor of diacylglycerides. *Nature* 433, 523-527

4 Jiang,Z. *et al.* (2005) CD14 is required for MyD88-independent LPS signaling. *Nat. Immunol.* 6, 565-570

5 Brown,G.D. (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 6, 33-43

6 Dennehy,K.M. and Brown,G.D. (2007) The role of the beta-glucan receptor Dectin-1 in control of fungal infection. *J. Leukoc. Biol.* 82, 253-258

7 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

8 Gross,O. *et al.* (2006) Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442, 651-656

9 Rogers,N.C. *et al.* (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity.* 22, 507-517

10 LeibundGut-Landmann,S. *et al.* (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8, 630-638

11 Underhill,D.M. *et al.* (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* 106, 2543-2550

12 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

13 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

14 Herre,J. *et al.* (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104, 4038-4045

15 Williams,D.L. *et al.* (1991) A method for the solubilization of a (1----3)-beta-D-glucan isolated from Saccharomyces cerevisiae. *Carbohydr. Res.* 219, 203-213

16 Covert,M.W. *et al.* (2005) Achieving stability of lipopolysaccharide-induced NF-kappaB activation. *Science* 309, 1854-1857

17 Gautier,G. *et al.* (2005) A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J. Exp. Med.* 201, 1435-1446

18 Napolitani,G. *et al.* (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6, 769-776

19 Bagchi,A. *et al.* (2007) MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol.* 178, 1164-1171

20 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

21 Sanjuan,M.A. *et al.* (2006) CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. *J. Cell Biol.* 172, 1057-1068

22 Hamerman,J.A. *et al.* (2005) Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat. Immunol.* 6, 579-586

23 Bleharski,J.R. *et al.* (2003) A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J. Immunol.* 170, 3812-3818

24 Gerber,J.S. and Mosser,D.M. (2001) Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J. Immunol.* 166, 6861-6868

25 Suzuki-Inoue,K. *et al.* (2006) A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood* 107, 542-549

26 Chaipan,C. *et al.* (2006) DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets. *J. Virol.* 80, 8951-8960

27 Chen,T. *et al.* (2001) The CGM1a (CEACAM3/CD66d)-mediated phagocytic pathway of Neisseria gonorrhoeae expressing opacity proteins is also the pathway to cell death. *J. Biol. Chem.* 276, 17413-17419

28 McCaw,S.E. *et al.* (2003) Immunoreceptor tyrosine-based activation motif phosphorylation during engulfment of Neisseria gonorrhoeae by the neutrophil-restricted CEACAM3 (CD66d) receptor. *Mol. Microbiol.* 49, 623-637

29 Hara,H. *et al.* (2007) The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat. Immunol.* 8, 619-629

30 Suram,S. *et al.* (2006) Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor. *J. Biol. Chem.* 281, 5506-5514

31 Marshall,A.S. *et al.* (2006) Human MICL (CLEC12A) is differentially glycosylated and is down-regulated following cellular activation. *Eur. J. Immunol.* 36, 2159-2169

32 Dennehy,K.M. *et al.* (2003) Mitogenic signals through CD28 activate the protein kinase Ctheta-NF-kappaB pathway in primary peripheral T cells. *Int. Immunol.* 15, 655-663

Chapter 4

A novel functional screening approach identifies CD47 as a target of dectin-1 induced stimulation of macrophages

Gerben Ferwerda, Sigrid E. Heinsbroek, Mihai G. Netea and Siamon Gordon

Submitted

Abstract

Dectin-1 is a C-type lectin expressed on myeloid cells and is a pattern recognition receptor for β-glucans. The role of dectin-1 for the modulation of membrane-bound proteins involved in immune responses is not known. We have employed a functional approach to screen for the effect of dectin-1 engagement on the expression of membrane proteins by stimulating murine peritoneal macrophages overnight with zymosan. Changes in the expression selected membrane proteins were analyzed by FACS. To determine the role of dectin-1 in this process we compared macrophages isolated from dectin-1+/+ (WT) and dectin-1-/- macrophages. There was a distinct pattern of up- and down-regulation of proteins indicating a specific role in the immune response. The integrin-associated protein CD47 was specifically down-regulated by zymosan in a dectin-1 dependent manner. This effect was independent on zymosan–induced phagocytosis, while no effect on cell death/apoptosis was apparent. Incubation with β-glucan alone or HK *C. albicans* lowered the expression of CD47, whereas incubation with LTA (a TLR2 agonist) had no effect on the expression of CD47. These experiments demonstrate that screening membrane bound proteins by FACS after overnight stimulation with zymosan in dectin-1 -/- and WT macrophages can reveal novel interactions between dectin-1 and membrane-bound molecules. Dectin-1 stimulation with β-glucan leads to a lower expression of CD47 on murine macrophages, and this could play a role in the cross-presentation fungal antigens.

Introduction

The human body is colonized with microorganisms that represent our commensal microbiological flora [1,2]. The epithelial barrier is crucial in maintaining the balance between colonization and invasion by these organisms [3,4]. The epithelial cells and a complex network of immune cells located underneath the epithelial layer are capable to recognize the invading organism and induce an immune response to eliminate the pathogen without causing collateral damage the surrounding tissue [5]. The residential macrophages and dendritic cells (DC) are both important antigen presenting cells (APC) in this network, of which the latter is the strongest inducer of the acquired immune response by migrating to the local lymph nodes and presenting the antigen to the T-cells [6,7]. The macrophages are less mobile and highly adapted to the local environment and regulatory function of the epithelial (and mucosal) immunological homeostasis [7,8].

Fungi and yeast are part of the commensal microbiological flora of humans, of which *Candida albicans* can cause disease when invading the host [9-11]. Macrophages play a crucial role in the host defense against *Candida* [12]. Pathogen receptors of the Toll-like receptor (TLR) and lectin-like receptor (LR) family on the membrane of the macrophages can recognize cell wall components of *C. albicans* and activate different pathways leading to cytokine production, phagocytosis and antigen presentation [13]. The induction of cytokines and chemokines will activate and attract to the site of infection other immune cells like neutrophils and T-cells [14]. After phagocytosis, *Candida* can be killed intracellular by lysosomal enzymes and reactive oxygen species (ROS) [15]. The macrophage also functions as an antigen presenting cell, capable to activate and regulate the acquired immune response [16,17]. In order to activate T-cells, cytokines and expression of co-stimulatory molecules and direct cell contact is necessary [18,19]. Therefore, beside 'communication' through secretion of proteins, like cytokines , direct cell-cell interaction through membrane bound proteins play an important role in the regulatory function of macrophages.
Very little is known regarding the expression pattern of membrane-bound proteins that determine the function of macrophages upon cell-cell interactions during stimulation with *C. albicans*. One of the main recognition receptors for Candida is the β-glucan receptor dectin-1 [20,21]. We investigated in murine macrophages the dectin-1 induced modulation in the expression of membrane-bound proteins involved in immune responses. By screening with a large panel of anti-bodies in a flow cytometry assay we show that incubation with zymosan (cell wall components of *Saccharomyces cerevisiae*, containing β-glucan and TLR2 ligands) leads to a distinct pattern of up- and down- regulation of membrane-bound proteins. We further focused on the protein CD47, as this protein was specifically down regulated after stimulation of dectin-1 with β-glucan, and postulate that this might play a role in cross-presentation of fungal antigens.

Results

CD47 is identified as a target of dectin-1-dependent stimulation of murine macrophages

To investigate the changes in the expression of membrane bound proteins on murine macrophages after incubation with zymosan, we used a broad panel of antibodies against proteins involved in immunological processes. This resulted in a distinct pattern of modulation of these proteins (supplemental table 1). To further investigate the specific role of dectin-1 in this process we compared the expression of proteins in dectin-1 defective and WT macrophages. There were significant differences in up-regulation of CD25, CD80, CD86 and PD-L1 and down regulation of PDL2, CD95 and CD47 (Fig 1 and supplemental table 1). To validate our screening method we focused in on CD47 because the inhibition of CD47 expression by dectin-1 is a novel function of the receptor.

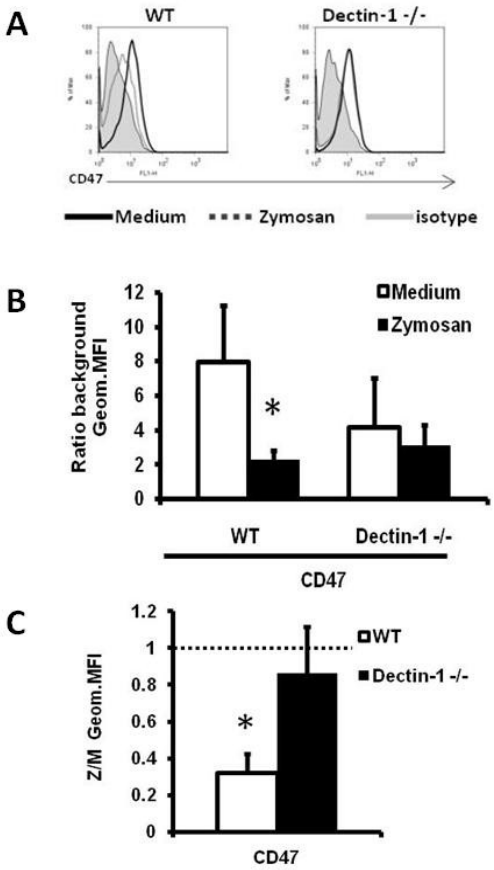

Figure 1. CD47 expression on murine macrophages is reduced after stimulation with zymosan. Biogel-elicited peritoneal macrophages were stimulated overnight with zymosan (5 particles per cell) or medium. For each experiment macrophages of 5 mice were pooled before stimulation and the expression of CD47 was measured by FACS analysis (**Panel A**). 5 independent experiments were performed and the data are expressed either as ratio CD47/rIgG2a of the geometric mean fluorescence (Geom. MFI) (**Panel B**) or ratio of CD47 expression after zymosan and medium stimulation (**Panel C**). Data presented as mean ± SD and compared with Student's t-test (* $p < 0.05$)

There is no difference in apoptosis between dectin-1 -/- and WT murine macrophages after stimulation with zymosan

Because low expression of CD47 may be explained by apoptosis of macrophages induced by zymosan, we first determent the level of apoptosis by measuring the uptake of rhodamine 123 as marker of early apoptosis and 7-amino-actinomycin D (7-AAD) as marker of late apoptosis. There was no difference in apoptosis between dectin-1 -/- and WT murine macrophages after stimulation with zymosan (Fig 2A and B). This indicates that down regulation of CD47 in WT macrophages after stimulation with zymosan is not induced by induction of apoptosis by dectin-1.

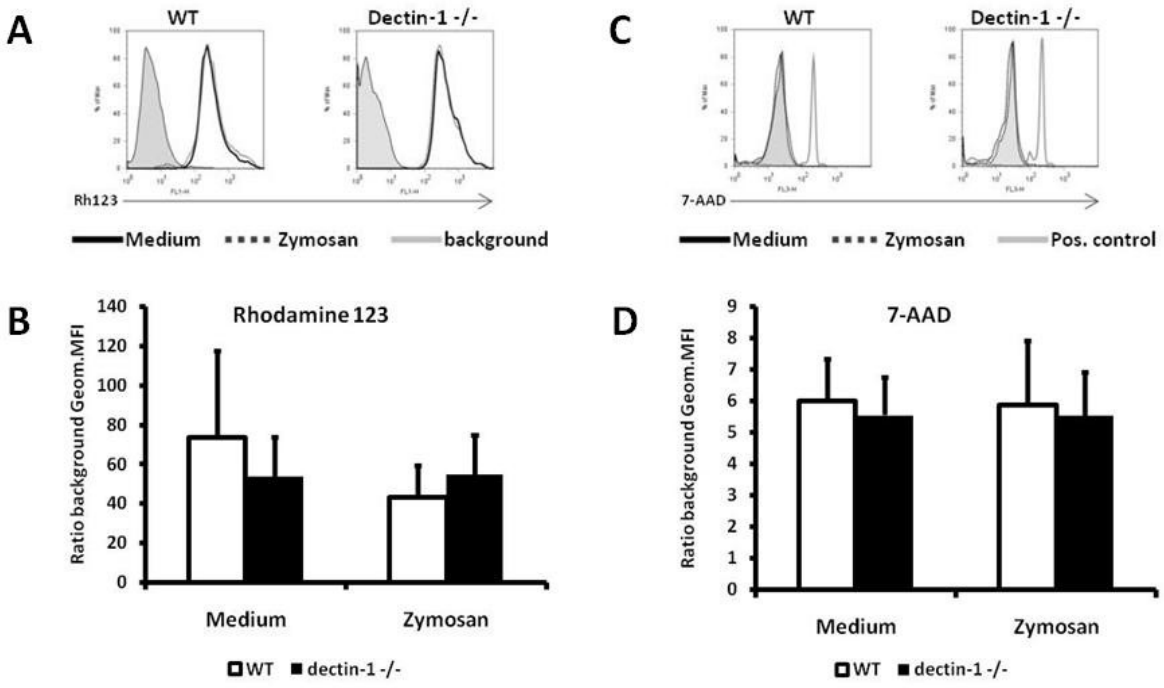

Figure 2. *No* differences in apoptosis between WT and dectin-1 -/- macrophages after incubation with zymosan. Biogel-elicited peritoneal macrophages were stimulated with zymosan (5 particles per cell) or medium overnight and degree of apoptosis was measured by rhodamine123 (**Panel A and B**) and 7-AAD (**Panel C and D**) by FACS analysis. For each experiment macrophages of 5 mice were pooled and experiments performed twice independently. Data expressed as ratio rhodamine 123 /background of the geometric mean fluorescence (Geom. MFI) and presented as mean ± SD and compared to medium by Student's t-test (* $p < 0.05$)

Down-regulation of CD47 after stimulation with zymosan is induced within hours and is dose dependent

To further characterize the modulation of CD47expression by zymosan, the time curve of the modulation was assessed (Fig 3A). Within the first two hours the expression of CD47 was down-regulated and maintained at the same level for the first 12 hours; in the next 12 hours there is a slightly but significant reduction of CD47. In the dectin-1 -/- macrophages the CD47 expression is not reduced after incubation with zymosan for 24 hours. The down regulation of CD47 is dose dependent (Fig 3B). This data points out to a sustained role for CD47 down-regulation in the early immune response to zymosan.

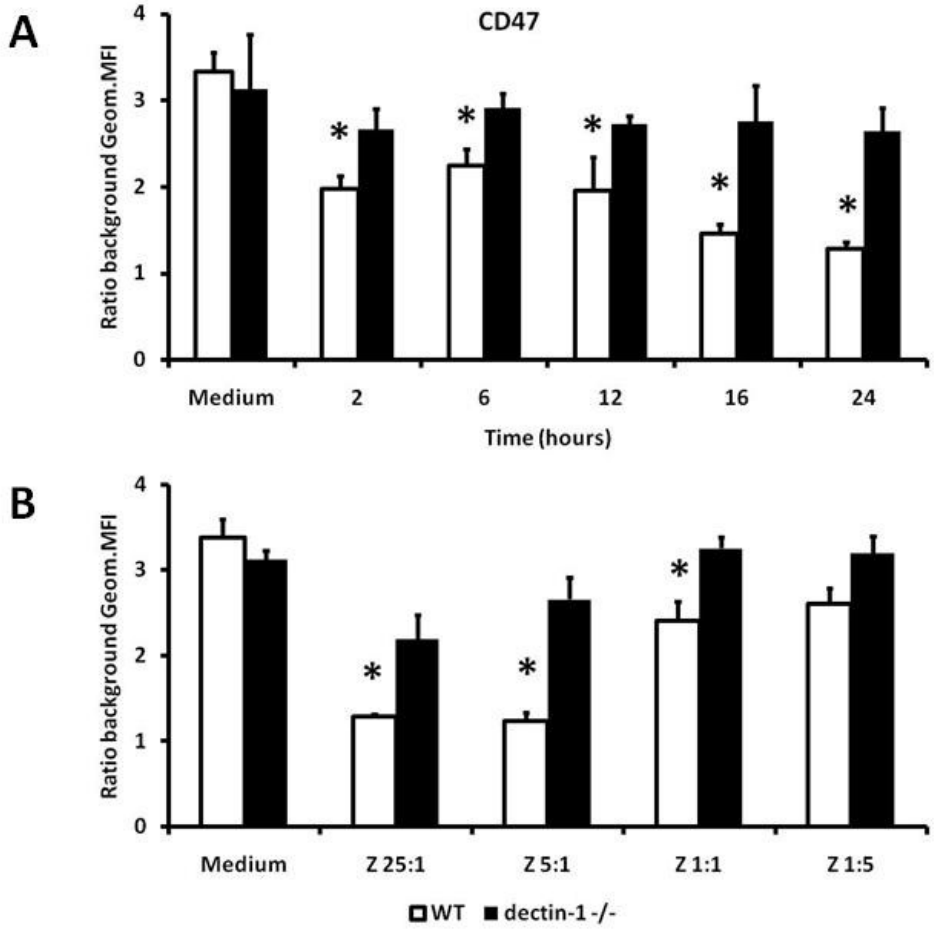

Figure 3. The reduction of CD47 expression after stimulation with zymosan is an early event and dose dependent. Biogel-elicited peritoneal macrophages were stimulated with zymosan (5 particles per cell) or medium and CD47 expression was measured at different time points with FACS analysis (**Panel A**). Dose response curve after overnight stimulation with zymosan (particles per cell) (**Panel B**). For each experiment macrophages of 5 mice were pooled and experiments performed twice independently. Data expressed as ratio CD47/rIgG2a of the geometric mean fluorescence (Geom. MFI) and presented as mean ± SD and compared to medium by Student's t-test (* $p < 0.05$)

Down-regulation of CD47 is largely independent of zymosan phagocytosis

Dectin-1 is involved in binding of zymosan which induces both a cytokine response and phagocytosis. Inhibiting phagocytosis of zymosan with cytochalasin D did not prevent down regulation of CD47 in WT macrophages, although slightly reduced it (Fig 4A). This indicates that engagement of zymosan by dectin-1 leads to reduction of CD47 expression largely independent of phagocytosis.

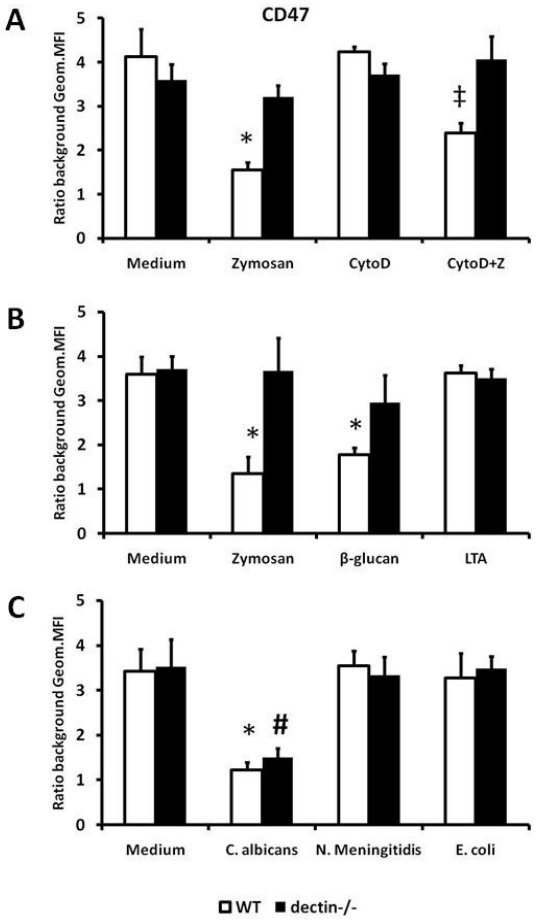

Figure 4. The down regulation of CD47 on macrophages after stimulation with zymosan is phagocytosis independent, TLR2 independent and dectin-1 dependent. Biogel-elicited peritoneal macrophages were stimulated with 5 particles zymosan per cell (Z) or medium after 30 minutes pre-incubation with 10 µg/ml cytochalasin D (CytD) (**panel A**). Macrophages were stimulated with particulated β-glucan (5 particles per cell) and LTA (10 µg/ml) (**panel B**). Macrophages were stimulated with heat killed *C. albicans* (5 conidia per cell), heat killed *N. Meningitidis* (50 per cell) and *E. coli* (50 per cell) (**Panel C**). CD47 expression was measured with FACS analysis after overnight stimulation. For each experiment macrophages of 5 mice were pooled and experiments performed twice independently. Data expressed as ratio CD47/rIgG2a of the geometric mean fluorescence (Geom. MFI) and presented as mean ± SD. Expression was compared with Student's t-test to medium (* and # $p < 0.05$) and to cytochalasin D (‡ $p < 0.05$).

Engagement of β-glucan by dectin-1 leads to down-regulation of CD47

Zymosan consists cell-wall particles of *Saccharomyces cerevisiae*, rich of β-glucan, but also other components which induce TLR2 mediated immune responses [22,23]. To further determine the specific role of dectin-1 in the down regulation of CD47 after stimulation with zymosan, we in incubated murine macrophages with purified β-glucan or with the specific TLR2 ligand lipoteichoid acid (LTA). β-glucan reduced CD47 expression in the WT macrophages but not in dectin-1-/- macrophages. Stimulation with TLR2 ligand did not reduce the CD47 expression in WT or dectin-1 -/- macrophages (Fig 4B). Incubation of the macrophages with heat killed *Candida albicans* resulted in a reduction of membrane bound CD47 in both dectin-1 -/- and WT macrophages (Fig 4B). The data imply that binding of β-glucan to dectin-1 leads to down regulation of CD47. However, *Candida albicans* stimulation of macrophages activates redundant mechanisms that also lead to the reduction of CD47 expression.

Discussion

In this study we assessed a novel approach for identification of biological functions of pattern recognition receptors for changes in the expression of membrane-bound proteins after incubation of the target cells with specific ligands. Using the β-glucan rich particle zymosan in WT and dectin-1 -/- murine macrophages, we identified CD47 as a novel target of dectin-1 induced stimulation of macrophages.

There is little insight in the modulation of protein expression on the membrane of macrophages during engagement of pattern recognitions receptors (PRRs) by their specific ligands [24,25]. Screening for changes in expression of proteins by FACS analysis has the big advantage that it immediately leads to data pertaining PRR function, especially when compared to knock-out strains. By using an array of antibodies against membrane-bound proteins, novel functional effects of PRRs can be identified. Our screening using stimulation of murine peritoneal macrophages with the fungal particle zymosan, resulted in a distinct pattern of down-regulation of proteins (CR3, CD11b, CD210, CD229, CD45RB, CD95, ICOS-L, PD-1, PD-L2 and FCRII/III) and up-regulation of proteins (CD25, CD80, CD86 and CD200). Because we were interested in the role of dectin-1 in the regulation of membrane bound molecules, we compared the effect of zymosan on protein expression in macrophages isolated from WT and dectin-1 -/- mice. This led to a limited number of molecules of interest. The difference in expression of CD80 and CD86 after stimulation with β-glucan has already been reported in the literature [20,26,27]. The differences in expression of PD-L1 and PD-L2 have not yet been described in fungal infection, although it has been reported that microparticulate β-glucan can up regulate PD-L1 [27]. The inhibition of the expression of CD95 (Fas) could be involved in the immunological response to fungal infection, because Fas-FasL interaction modulate cytokine responses, and MRL/lpr mice lacking the Fas molecule are more resistant to *Candida* than the WT [28].
One of the most constant effects of dectin-1 stimulation was the down-regulation of CD47 expression. There are no reports on the role of CD47 in fungal or mucosal infection, although its role in infections has been described in mice. The first reports on CD47 -/- mice show that these mice had a decreased resistance to bacterial infection caused by an impaired granulocyte function [29,30]. The most studied role of CD47 is the induction of phagocytosis of apoptotic cells. CD47 is expressed on all cells and low expression leads to phagocytosis [31], as shown in the clearance of erythrocytes [32,33]. The interaction of CD47 and its ligand Sirp-α maintain a 'don't eat me' signal, and disruption of this interaction induces phagocytosis [31,34]. The lower expression of CD47 is also involved in the controlled phagocytosis of viable cells during infection and inflammation. Gardai et al show that calreticulin/LRP interaction can induce engulfment of infected cells if CD47 expression is reduced [35]. This controlled phagocytosis of viable cells may play a role in cross presentation of antigens [36,37]. Other reports have shown that the expression of CD47 is also involved in the mobility of dendritic cells (Langerhands), and when the CD47 expression declines these cells are less mobile [38-40].

In the experiments we present here we show that the difference in CD47 expression could not be explained by difference in cell apoptosis. In addition, phagocytosis played a minor role (if any) in the dectin-1 mediated down-regulation of CD47. This indicates direct effects on CD47 expression after engagement of zymosan by dectin-1.
To point out the specificity of our findings we repeated the experiment with highly purified β-glucan, resulting in the same extent of down regulation of CD47 compared to zymosan. In contrast, stimulation of cells with a TLR2 ligand had no effect on CD47 expression. The potential importance of the CD47 expression inhibition is also suggested by the redundancy when dectin-1 -/- macrophages were incubated with whole *Candida albicans*. Although there is difference in binding and phagocytosis of Candida by dectin-1 -/- macrophages [20], activation of other receptor-pathways resulted in the down regulation of CD47 expression in dectin-1 -/- cells comparable to WT macrophages. This suggests that CD47 inhibition is an important component of the interaction of innate host defense with fungal pathogens. This is supported by the observation that the inhibition of CD47 is specific for a fungal pathogen such as *C. albicans*, while the Gram-negative bacteria *N. meningitidis* and *E. coli* do not modulate CD47 expression.

The role of macrophages in fungal infection is multifaceted. On the one hand, macrophages function as phagocytic cells at the site of infection, with an active role in the clearance and killing of microorganisms like *Candida albicans*. On the other hand, macrophages function as gatekeeper and regulator in tissues like the mucosa. The activation of macrophages by fungal particles induces a specific immune response shown by the changes in membrane bound proteins involved in immunity. The down regulation of CD47 indicates a regulated phagocytosis-inducing signal. This consequence of dectin-1 engagement is also likely important for cross-presentation of the phagocytosed fungal particles, but also a marker for cellular mobility, signaling to the resident macrophages to stay in the tissue to which it has been adapted and function as a local regulatory myeloid cell. In this process, pathogen recognition receptors like dectin-1 seem to play an important role.

Supplemental table 1

CD nr	Alternative name	Function	WT Medium	WT Zymosan		dectin-1 -/- Medium	dectin-1 -/- Zymosan		WT Z/M	dectin-1 -/- Z/M	Catalog nr	Clone	Company
CD4	T4, Leu3a	MHC class II coreceptor, T cell differentiation/activation	1.66	1.15		1.56	1.42		0.71	0.96	*553047*	**L3T4**	BD
CD8a	T8, Leu-2	MHC class I coreceptor, T cell differentiation/activation	2.13	1.72		1.78	1.63		0.95	0.97	*553035*	**LY-2(53-6.7)**	BD
CD9	p24, MRP-1	cellular adhesion and migration	1.81	2.56		2.02	2.87		1.47	1.40	*11-0091-81*	**KMC8**	EB
CD11b	Mac-1, integrin aM	binds CD54, ECM, iC3b	152.88	77.44	*	159.71	91.89	*	0.51	0.62	*553312*	**M1/79**	BD
CD11b	Mac-1, integrin aM	binds CD54, ECM, iC3b	7.12	4.61	*	6.44	4.45	*	0.67	0.69	*MCA711FT*	**5C6**	ST
CD11c	CR4, integrin	binds CD54, fibrinogen and iC3b	2.05	1.81		1.85	1.94		1.00	1.09	*553802*	**HL3**	BD
CD25	Tac, p55	IL-2Ra, w/ IL-2Rb and g to form high affinity complex	1.44	2.08	*	1.59	1.78		1.48	1.11	*553071*	**7D4**	BD
CD27	T14	CD70 receptor, T costimulation	0.82	0.93		0.84	1.03		1.14	1.23	*556754*	**LG.3A10**	BD
CD38	T10	ecto-ADP-ribosyl cyclase, cell activation, cyclase & hydrolase activity	13.35	7.64	*	11.22	5.18	*	0.57	0.46	*553764*	**90**	BD
CD40	TNFRSF5	CD154R, B differentiation/costimulation, isotype-switching, rescues B cells from apoptosis	0.92	0.97		0.78	0.92		1.12	1.21	*553790*	**(3/23)**	BD
CD45RB	LCA	exon B isoform CD45, tyrosine phosphatase, enhanced TCR & BCR signals	5.36	2.77	*	2.90	2.08	*	0.76	0.73	*553101*	**16A**	BD
CD47	IAP	leukocyte adhesion, migration, activation, thrombospondin receptor	5.43	1.81	*	3.03	2.46		0.37	0.89	*555298*	**MIAP301**	BD
CD62L	L-selectin, LECAM-1	CD34, GlyCAM, and MAdCAM-1 receptor, leukocyte homing, tethering, rolling	2.02	1.90		1.21	1.27		0.94	1.05	*553151*	**MEL-14**	BD
CD63	LIMP, LAMP-3	lysosomal membrane protein, moves to cell surface after activation	1.98	1.70		1.62	1.95		0.86	1.20	*D263-3*	**R5G2**	MBL
CD69	AIM	signal transduction	2.76	1.61		2.25	1.52		0.58	0.67	*557392*	**H1.2F3**	BD
CD70	Ki-24	CD27 ligand, T and B cell costimulation	1.67	1.55		1.46	1.26		0.93	0.86	*555286*	**FR70**	BD
CD80	B7, B7-1, BB1	binds to CD28, CD152, T costimulation	2.50	4.75	*	2.80	2.91		2.11	1.04	* *553768*	**16-10A1**	BD
CD86	B70, B7-2	binds to CD28, CD152, T costimulation	1.72	2.26	*	2.38	2.24		1.33	0.99	* *553691*	**GL-1**	BD
CD95	Apo-1, Fas	FasL (CD178) receptor, apoptosis	4.55	1.77	*	3.77	2.57	*	0.37	0.68	* *554258*	**Jo2**	BD
CD103	HML-1, integrin aE	w/ integrin b7, binds E-cadherin, lymph homing/retention	1.27	0.94		1.04	1.02		0.75	0.98	*557495*	**M290**	BD
CD137	4-1BB	T costimulation	1.05	0.85		0.89	1.04		0.81	1.17	*558976*	**4-1BB**	BD
CD152	CTLA-4	CD80 and CD86 receptor, negative regulation of T cell costimulation	0.80	1.01		0.90	1.02		1.26	1.13	*553720*	**UC10-4F10-11**	BD
CD154	CD40L	CD40 ligand, B and DC costimulation	1.12	1.33		0.94	1.12		1.25	1.19	*553658*	**MR1**	BD
CD178	FasL, CD95L	CD95 ligand, apoptosis, immune privilege, soluble form in serum NOK	1.28	1.46		1.23	1.58		1.14	1.28	*555293*	**MFL3**	BD
CD200	OX-2	inhibition of immune response	0.98	1.76	*	0.94	1.48	*	1.80	1.54	*hybridoma*	**OX90**	SWDSOP
CD200R	OX-2L	ligand of CD200	1.24	1.22		1.10	0.95		1.04	0.92	*hybridoma*	**OX110**	SWDSOP
CD210	IL-10-R	IL-10 receptor, signal transduction	2.07	1.32	*	1.97	1.48	*	0.64	0.75	*559914*	**1B1.3A**	BD
CD229	Ly-9	adhesion	3.42	2.69	*	3.69	2.93	*	0.79	0.79	*553117*	**30C7**	BD
CD252	OX-40L	T costimulation	1.81	1.32		1.44	1.25		0.73	0.87	*12-5905-81*	**RM134L**	EB
CD273	B7DC, PD-L2	PD-1 receptor, costimulation or suppression of T proliferation	2.35	1.44	*	1.59	1.48		0.62	0.93	* *12-5986-81*	**TY25**	EB
CD274	B7-H1, PD-L1	PD-1 receptor, costimulation of lymphocytes	17.42	12.50	*	7.08	12.94	*	0.73	1.89	* *12-5982-81*	**MIH5**	EB
CD275	B7-H2, ICOSL	costimulation, cytokine production	2.87	1.95	*	1.80	1.51	*	0.67	0.85	*12-5985-81*	**HK5.3**	EB
CD279	PD1	B7-H1 & B7-DC receptor, autoimmune disease and peripheral tolerance	9.83	5.72	*	6.08	4.46	*	0.65	0.69	*12-9985-81*	**J43**	EB
CD314	NKG2D, KLR	binds MHC class I, activates cytolysis and cytokine production, costimulation	2.36	1.54		1.34	1.66		0.65	1.24	*12-5882-81*	**2G9**	EB
MHCII	MHCII	major histocompitibility complex, antigen presentation	1.89	1.92		2.33	2.09		1.03	0.91	*MCA2400A488*	**ER-TR2**	ST
I-Ad/I-Ed	MHC II alloantigens	major histocompitibility complex, antigen presentation	5.70	3.69		3.13	2.51		0.65	0.86	*553623*	**2G9**	BD
CD16/32	FcγRII/III	component of low affinity Fc receptor, phagocytosis and ADCC	2.15	1.24	*	1.75	1.12	*	0.58	0.64	*hybridoma*	**24G2**	SWDSOP
FoxP3	Forkhead Box P3	marker for t-cell differentiation	1.45	0.88		1.16	1.14		0.61	0.98	*12-5773-80*	**FJK-16S**	EB
Gr-1	Ly-6G and Ly-6C	granulocyte and monocyte differentiation and maturation marker	4.80	3.25		4.05	5.15		0.70	1.27	*553127*	**RB6-8C5**	BD
F4/80	F4/80	marker for macrophage activation (type I)	2.08	1.52		1.37	1.29		0.73	0.95	*MCA497FB*	**A3-1**	ST
LPAM-1	Integrin α4β7 complex	interacts with several ligands, including VCAM-1 (CD106), fibronectin, and MAdCAM-1	1.84	1.01		1.84	1.37		0.55	0.74	*553811*	**DATK32**	BD
MARCO	MARCO	class A scavenger receptor	0.75	0.87		0.67	0.97		1.15	1.57	*MCA1849FT*	**ED31**	ST

Ratio background Geom.MFI

Ratio Zymosan/Medium

Materials and methods

Isolation and stimulation of murine macrophages

The mice used in this study (129/SvEv Clec7+/+ and Clec7-/-) were bred in our own institutional colonies. Sex- and age-matched (between 8 and 12 wk of age) were used in the study. Animals were kept and handled in accordance with institutional guidelines. All experiments were approved by the ethical committee on animal experiments of Oxford University.

Biogel elicited peritoneal macrophages were prepared by i.p. injection of 1 ml polyacrylamide gel P-100 (Bio-Rad) beads (2% w/v in endotoxin-free water). After 4 days, peritoneal cells were harvested by lavage with phosphate-buffered saline (PBS). The macrophages were plated on BP dishes in a defined serum-free medium, OPTIMEM, supplemented with 50 IU/mL penicillin-streptomycin and 2 mM L-glutamine. After 3-4 h, adherent cells were washed three times to remove non-adherent cells and bio-gel beads. For the screening experiments $10x10^6$ cells were plated on BP dishes and stimulated with zymosan (Sigma-Aldrich) overnight. For all the other experiments $1.5x10^6$ cells were plated in 6 wells plates.

Inhibiting phagocytosis was obtained by pre-incubation for 30 minutes with 10 µg/ml cytochalasin D (Sigma-Aldrich). The particulated β-glucan was a kind gift of D.L Williams (Department of Surgery, James H. Quillen College of Medicine, Johnson City, USA) and used at 5 particles per cell. LTA was purchased from Sigma Aldrich and used at 10 µg/ml. Heat killed *Candida albicans* (SC 5314) was obtained according previous described [41] and used at 5 conidia per cell. *Neisseria Meningitidis* (MC58) and Escherichia coli (K1) were prepared as described by Peiser et al. [42,43] and used at 50 bacteria per cell.

Flow cytometry assays

After overnight stimulation the cells were washed 3 times with PBS and detached by incubation for 15 minutes in lidocaine/EDTA on ice and thereafter by scraping. The detached cells were washed in 50 ml PBS and centrifuged at 1200 rpm, and the pallet was incubated in blocking buffer (5% heat-inactivated rabbit serum, 0.5% BSA, 5 mM EDTA, and 2 mM NaN_3) for 30 min at 4°C. Cells were counted and divided in V-shaped 96 wells plates at $5x10^5$ cells per well. Cells were incubated with primary antibodies for 1 h at 4°C in blocking buffer and washed three times with a washing buffer containing 0.5% BSA, 5 mM EDTA, and 2 mM NaN_3. If necessary, secondary antibody was incubated with the cells for 1 h at 4°C in blocking buffer, and the cells were washed three times as described above and fixed in 1% formaldehyde. Expression patterns were analyzed on a FACSCalibur using CellQuest software.

The primary antibodies used in this study were produced in the Sir William Dunn School of Pathology in Oxford (SWDSOP) or obtained from BD Pharmingen (BD), eBioscience (EB), Serotec (ST) or Medical and Biological Laboratories Co. (MBL) as indicated in the supplemental table 1. Isotype controls were from BD Pharmingen and were matched to the isotype of the primary anti-bodies used in each experiment.

Apoptosis measurement

Cells were counted and divided in V-shaped 96 wells plates at $5x10^5$ cells per well. Early apoptosis was measured by mitochondrial staining with 200 nM rhodamine 123 (Sigma - Aldrich) for 20 minutes at 37°C in the dark and washed 3 times in blocking buffer and analyzed by FACS. Late apoptosis was measured by DNA staining with 100 µg/ml 7-amino-actinomycin D (Sigma-Aldrich) for 30 minutes at 4°C in the dark and washed in blocking buffer before FACS analysis.

Statistical analysis

Data obtained with FACS analysis was expressed as geometric mean fluorescence intensity (Geom.MFI). This data was compared to the background obtained by the aspecific binding of isotype matched control antibodies and shown as ratio Geom. MFI specific antibody/Geom. MFI isotype control. Differences between dectin-1 -/- and WT macrophages was calculated with the Student's t-test. Level of significance was set at $p < 0.05$.

Reference List

1 Pamer,E.G. (2007) Immune responses to commensal and environmental microbes. *Nat. Immunol.* 8, 1173-1178

2 Backhed,F. *et al.* (2005) Host-bacterial mutualism in the human intestine. *Science* 307, 1915-1920

3 Artis,D. (2008) Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat. Rev. Immunol.* 8, 411-420

4 Macdonald,T.T. and Monteleone,G. (2005) Immunity, inflammation, and allergy in the gut. *Science* 307, 1920-1925

5 Macpherson,A.J. and Harris,N.L. (2004) Interactions between commensal intestinal bacteria and the immune system. *Nat. Rev. Immunol.* 4, 478-485

6 Sigmundsdottir,H. and Butcher,E.C. (2008) Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. *Nat. Immunol.* 9, 981-987

7 Platt,A.M. and Mowat,A.M. (2008) Mucosal macrophages and the regulation of immune responses in the intestine. *Immunol. Lett.* 119, 22-31

8 Smythies,L.E. *et al.* (2005) Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J. Clin. Invest* 115, 66-75

9 Lott,T.J. *et al.* (2005) The human commensal yeast, Candida albicans, has an ancient origin. *Fungal. Genet. Biol.* 42, 444-451

10 Edmond,M.B. *et al.* (1999) Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Infect. Dis.* 29, 239-244

11 Reef,S.E. *et al.* (1998) Nonperinatal nosocomial transmission of Candida albicans in a neonatal intensive care unit: prospective study. *J. Clin. Microbiol.* 36, 1255-1259

12 Vazquez-Torres,A. and Balish,E. (1997) Macrophages in resistance to candidiasis. *Microbiol. Mol. Biol. Rev.* 61, 170-192

13 Netea,M.G. *et al.* (2008) An integrated model of the recognition of Candida albicans by the innate immune system. *Nat. Rev. Microbiol.* 6, 67-78

14 Musso,T. *et al.* (1998) Interleukin-15 activates proinflammatory and antimicrobial functions in polymorphonuclear cells. *Infect. Immun.* 66, 2640-2647

15 Netea,M.G. *et al.* (2004) Human dendritic cells are less potent at killing Candida albicans than both monocytes and macrophages. *Microbes. Infect.* 6, 985-989

16 Redmond,H.P. *et al.* (1993) Macrophage-dependent candidacidal mechanisms in the murine system. Comparison of murine Kupffer cell and peritoneal macrophage candidacidal mechanisms. *J. Immunol.* 150, 3427-3433

17 Hume,D.A. (2008) Macrophages as APC and the dendritic cell myth. *J. Immunol.* 181, 5829-5835

18 Vignali,D.A. *et al.* (2008) How regulatory T cells work. *Nat. Rev. Immunol.* 8, 523-532

19 Leibson,P.J. (2004) The regulation of lymphocyte activation by inhibitory receptors. *Curr. Opin. Immunol.* 16, 328-336

20 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

21 Gow,N.A. *et al.* (2007) Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* 196, 1565-1571

22 Brown,G.D. *et al.* (2002) Dectin-1 is a major beta-glucan receptor on macrophages. *J. Exp. Med.* 196, 407-412

23 Underhill,D.M. (2003) Macrophage recognition of zymosan particles. *J. Endotoxin. Res.* 9, 176-180

24 Taylor,P.R. *et al.* (2005) Macrophage receptors and immune recognition. *Annu. Rev. Immunol.* 23, 901-944

25 Gordon,S. and Taylor,P.R. (2005) Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5, 953-964

26 Saijo,S. *et al.* (2007) Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat. Immunol.* 8, 39-46

27 Hunter,K.W., Jr. *et al.* (2004) Microparticulate beta-glucan upregulates the expression of B7.1, B7.2, B7-H1, but not B7-DC on cultured murine peritoneal macrophages. *Immunol. Lett.* 93, 71-78

28 Netea,M.G. *et al.* (1999) Fas-FasL interactions modulate host defense against systemic Candida albicans infection. *J. Infect. Dis.* 180, 1648-1655

29 Lindberg,F.P. *et al.* (1996) Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science* 274, 795-798

30 Verdrengh,M. *et al.* (1999) Integrin-associated protein (IAP)-deficient mice are less susceptible to developing Staphylococcus aureus-induced arthritis. *Microbes. Infect.* 1, 745-751

31 Kinchen,J.M. and Ravichandran,K.S. (2008) Phagocytic signaling: you can touch, but you can't eat. *Curr. Biol.* 18, R521-R524

32 Oldenborg,P.A. *et al.* (2000) Role of CD47 as a marker of self on red blood cells. *Science* 288, 2051-2054

33 Lindberg,F.P. *et al.* (1994) Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J. Biol. Chem.* 269, 1567-1570

34 Takizawa,H. and Manz,M.G. (2007) Macrophage tolerance: CD47-SIRP-alpha-mediated signals matter. *Nat. Immunol.* 8, 1287-1289

35 Gardai,S.J. *et al.* (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* 123, 321-334

36 Lin,J.S. *et al.* (2005) Dendritic cells cross-present exogenous fungal antigens to stimulate a protective CD8 T cell response in infection by Histoplasma capsulatum. *J. Immunol.* 174, 6282-6291

37 Megiovanni,A.M. *et al.* (2006) Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J. Leukoc. Biol.* 79, 977-988

38 Van,V.Q. *et al.* (2006) Expression of the self-marker CD47 on dendritic cells governs their trafficking to secondary lymphoid organs. *EMBO J.* 25, 5560-5568

39 de Vries,H.E. *et al.* (2002) Signal-regulatory protein alpha-CD47 interactions are required for the transmigration of monocytes across cerebral endothelium. *J. Immunol.* 168, 5832-5839

40 Hagnerud,S. *et al.* (2006) Deficit of CD47 results in a defect of marginal zone dendritic cells, blunted immune response to particulate antigen and impairment of skin dendritic cell migration. *J. Immunol.* 176, 5772-5778

41 Van der Graaf,C.A. *et al.* (2005) Differential cytokine production and Toll-like receptor signaling pathways by Candida albicans blastoconidia and hyphae. *Infect. Immun.* 73, 7458-7464

42 Peiser,L. *et al.* (2006) Identification of Neisseria meningitidis nonlipopolysaccharide ligands for class A macrophage scavenger receptor by using a novel assay. *Infect. Immun.* 74, 5191-5199

43 Peiser,L. *et al.* (2000) Macrophage class A scavenger receptor-mediated phagocytosis of Escherichia coli: role of cell heterogeneity, microbial strain, and culture conditions in vitro. *Infect. Immun.* 68, 1953-1963

Chapter 5

Human dectin-1 deficiency and mucocutaneous fungal infections

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Abstract

Mucocutaneous fungal infections are typically found in patients who have no known immune defects. We describe a family in which four women who were affected by either recurrent vulvovaginal candidiasis or onychomycosis had the early-stop-codon mutation Tyr238X in the β-glucan receptor dectin-1. The mutated form of dectin-1 was poorly expressed, did not mediate β-glucan binding, and led to defective production of cytokines (interleukin-17, tumor necrosis factor, and interleukin-6) after stimulation with β-glucan or Candida albicans. In contrast, fungal phagocytosis and fungal killing were normal in the patients, explaining why dectin-1 deficiency was not associated with invasive fungal infections and highlighting the specific role of dectin-1 in human mucosal antifungal defense.

Introduction

Recurrent vulvovaginal candidiasis is a relatively common pathological condition, afflicting women of all ages, with more than 90% of cases caused by *C. albicans* [1]. Although the role of diabetes as a predisposing condition has been recognized, most cases occur in healthy women. Recurrent oral, esophageal, or mucocutaneous candidiasis is also diagnosed in some patients without clear predisposing factors. Onychomycosis is a common infection of the nail beds, most often caused by dermatophytes but sometimes caused by *C. albicans*, that also affects immunocompetent persons [2]. In contrast, disseminated forms of candida infections are mostly found in patients in whom a defect in neutrophil function is easily recognized or candida species have been introduced in the bloodstream through invasive procedures [3]. Very little is known about the genetic factors predisposing patients to mucosal or disseminated candida infections.

Recognition of *C. albicans* by the innate host defense system is mediated by pattern-recognition receptors from the toll-like–receptor (TLR) and lectinlike-receptor families [4]. Mannans from the candida cell wall are recognized by the mannose receptor and TLR4 [5], and TLR2 recognizes phospholipomannan [6] and collaborates with the β-glucan receptor dectin-1 in the stimulation of cytokine production [7,8]. Recognition of 1,3-linked β-glucans by dectin-1 has been shown to be one of the main fungal-recognition pathways, and mice deficient in dectin-1 have an increased susceptibility to *C. albicans* [9] and *Pneumocystis carinii* [10]. Dectin-1 amplifies TLR2- and TLR4-induced cytokine production in both murine and human cells, resulting in the production of cytokines such as tumor necrosis factor [11], but also induces signals, independently of the TLRs, for the production of interleukin-17, interleukin-6, and interleukin-10 through a pathway dependent on spleen tyrosine kinase [12].

Results

Patients and pedigree

During the first phase of the screening, we identified a patient (the index patient) who had recurrent vulvovaginal candidiasis and had cells that were hyporesponsive to *C. albicans* stimulation, defined as cytokine production that was 15% or less of that stimulated by *C. albicans* in cells from healthy volunteers. The lack of cytokine production was pinpointed to an impaired response to β-glucan, indicating a potential defect in dectin-1 recognition.

To test this hypothesis, we sequenced the dectin-1 gene in the patient. All six exons of dectin-1 gene, and the nearby intronic regions, were sequenced (see Table 1 in the Supplementary Appendix). We identified a homozygous SNP in exon 6 that caused a change of amino acid 238 from tyrosine to a stop codon (Tyr238X), leading to the loss of the last nine amino acids of the carbohydrate-recognition domain (Figure 1A). Additional clinical questioning revealed that one of the patient's two sisters also had recurrent vulvovaginal candidiasis and both sisters had onychomycosis. The mother of the patient also had chronic onychomycosis, whereas the father had had only transient onychomycosis, with a relatively late age at onset and a complete recovery. The nucleotide sequence of wild-type dectin-1 in one healthy volunteer and the nucleotide change (AC) in exon 6 in two persons who were heterozygous or homozygous for the stop mutation are shown in Figure S3A in the Supplementary Appendix.

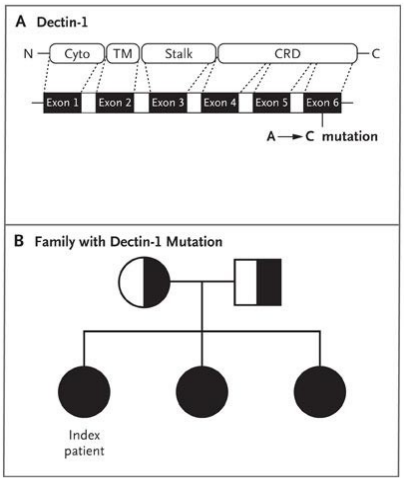

Figure 1. Dectin-1 mutation in a family with mucocutaneous fungal infections. Panel A shows the structure of the dectin-1 gene and the location of an early-stop-codon mutation consisting of a homozygous single-nucleotide polymorphism (rs16910526, exon 6, chromosome 12; location, 10162354 bp). The mutation (AC) causes a change of amino acid 238 from tyrosine to a stop codon (Tyr238X), leading to the loss of the last nine amino acids of the carbohydrate-recognition domain (CRD). Cyto denotes the cytoplasmic tail and TM the transmembrane region. Panel B shows the genetic pedigree of the index family with dectin-1 deficiency. Circles indicate female patients; the square indicates the father. Solid symbols denote homozygosity for the mutation (X/X, seen in all three daughters), and half-solid symbols denote heterozygosity (X/Tyr, seen in the parents).

The clinical characteristics of the patients are presented in Table 1. Microbiologic assessment of the nails of the three patients who were homozygous for the dectin-1 mutation revealed growth of *Trichophyton rubrum*. The patients had no known predisposing factors, such as diabetes mellitus or infection with the human immunodeficiency virus. Genetic analysis revealed that both sisters of the patient were homozygous for the Tyr238X mutation, whereas the parents were heterozygous (Figure 1B). The family members were white persons of Dutch ancestry, according to self-report, and the parents were not known to be related. Investigation of the human dectin-1 protein structure containing the early stop codon (Figure S3B in the Supplementary Appendix) revealed that a cysteine disulfide bridge between the helix and the deleted strand was absent, a finding likely to have important functional consequences.

Table 1. Clinical Characteristics of a Family of Patients with the Tyr238X Dectin-1 Mutation and with Mucocutaneous Fungal Infection, Onychomycosis, or Both.

Characteristic	Index Patient (Female)	Sister	Sister	Mother	Father
Dectin-1 nucleotide genotype*	X/X	X/X	X/X	X/Tyr	X/Tyr
Age (yr)	25	27	23	53	58
Age at onset of symptoms (yr)	10	10	12	40	55
Fingers and toes affected	All fingers and 4 toes, chronically	All fingers and toes, chronically	3 Fingers and 5 toes, transiently	All fingers and toes, chronically	4 Toes partially affected, with complete recovery
Recurrent vulvovaginal candidiasis	Yes	No	Yes	No	Not applicable

* X denotes the presence of a stop codon, rather than tyrosine (Tyr), at amino acid 238.

Table 1. Clinical characteristics of a family of patients with the Tyr238X dectin-1 mutation and with mucocutaneous fungal infection, onychomycosis, or both.

Immunologic defects

In the patients who had the early-stop-codon mutation, both monocytes (Figure 2A) and macrophages (not shown) had significant defects in the production of interleukin-6 after stimulation with β-glucan for 4 hours (P=0.04 for monocytes). Similar defects were apparent after stimulation of cells with either heat-killed or live *C. albicans* yeast (Figure S1A in the Supplementary Appendix). The cytokine response was also defective after 24 hours of stimulation with heat-killed *C. albicans* hyphae (with a 25% reduction of tumor-necrosis-factor production and a 34% reduction of interleukin-6 release). In addition, cells from dectin-1–deficient patients had a marked reduction in interleukin-17 production as compared with cells from persons with the dectin-1 wild-type allele (Figure S4A in the Supplementary Appendix). Heterozygotes had intermediate production of proinflammatory cytokines on stimulation with *C. albicans* or β-glucan. In contrast, the response of the patients' cells to TLR stimuli, such as lipopolysaccharide or lipopeptides, was normal (Figure S1B in the Supplementary Appendix). In addition, the amplification effect of the interaction between β-glucan and dectin-1 on TLR2 stimulation of cytokines [7] was absent in persons who were homozygous for the mutation (Figure S1C in the Supplementary Appendix).

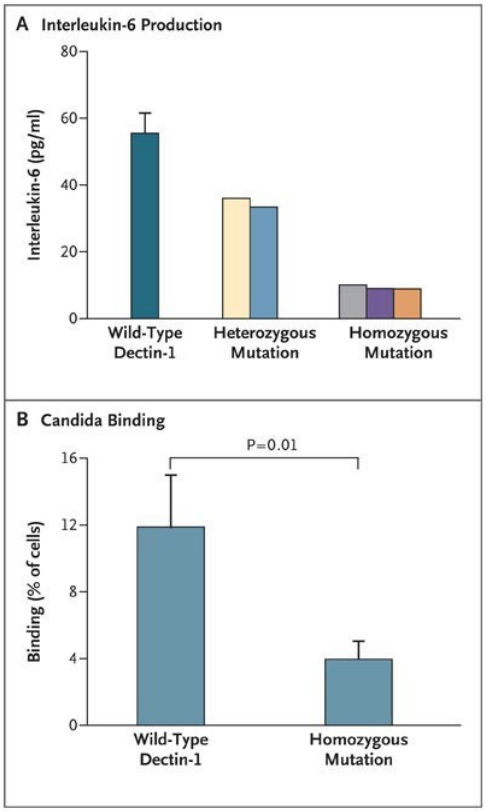

Figure 2. Functional defects resulting from dectin-1 mutation. Panel A shows interleukin-6 production in monocytes isolated after stimulation for 4 hours with β-glucan; for five healthy subjects with wild-type dectin-1 (with data pooled), the two patients who were heterozygous for the dectin-1 mutation, and the three patients who were homozygous for the mutation. Panel B shows the mean binding of nonopsonized fluorescent heat-killed *Candida albicans* to monocytes isolated from healthy subjects with wild-type dectin-1 and from the patients who were homozygous for the mutation. In both panels, T bars indicate standard deviations.

To demonstrate that the defect in β-glucan recognition in the patients was indeed due to the truncated dectin-1 variant, we constructed vectors for both wild-type and mutated isoforms A and B of dectin-1 and transfected them into NIH3T3 cells. These experiments revealed that neither mutated isoform A nor mutated isoform B could mediate β-glucan binding, in contrast to the wild-type receptors (Figure S4B in the Supplementary Appendix). Moreover, although the wild-type dectin-1 isoforms A and B were normally expressed, the mutated isoforms were associated with significantly lower expression on the cell surface of the transfected cells (Figure S4C in the Supplementary Appendix).

The in vitro data were corroborated in freshly isolated cells from the patients bearing the stop-codon mutation. Messenger RNA production by cells with the dectin-1 isoforms was similar in persons who were homozygous for the wild-type allele and the patients who were homozygous for the stop mutation (Figure S4D in the Supplementary Appendix). In contrast, monocytes and neutrophils from the patients who were homozygous for the stop-codon polymorphism lacked cell-membrane expression of dectin-1 (Figure S4E in the Supplementary Appendix). Heterozygotes had intermediate expression of dectin-1.

The binding of heat-killed fluorescence-labeled *C. albicans*, which has high levels of β-glucan [13], to monocytes was significantly lower in the patients who were homozygous for the mutation than in healthy controls (Figure 2B). However, phagocytosis of live *C. albicans* was normal in both monocytes and neutrophils from the patients who were homozygous for the stop codon (Figure S2 in the Supplementary Appendix), showing the importance of alternative receptors for phagocytosis of live yeasts (other lectinlike receptors and TLRs) [4]. Monocytes and neutrophils from the patients who were homozygous for the mutation were as effective at killing *C. albicans* as were cells from normal persons (Figure S2 in the Supplementary Appendix).

Frequency of the mutation in populations

To determine whether the dectin-1 stop mutation was occurring in a phylogenetically conserved site, we compared the amino acid sequence of dectin-1 among several mammals (Figure S5A in the Supplementary Appendix). This investigation revealed that the mutation lies in an evolutionarily conserved region within the mammalian lineage. We then investigated the prevalence of the mutation in various human populations, and we genotyped persons from four cohorts of healthy persons of various ethnic groups, each representing populations from the major continents: 138 whites of Dutch ancestry (Europe), 99 people from Tanzania (Africa), 100 Han Chinese (Asia), and 105 Trio Indians (Native Americans) from Surinam (South America). Dectin-1 was sequenced completely in the Dutch population, with Tyr238X being the only nonsynonymous mutation identified. The Dutch population had an allele frequency of 0.069, and the Tanzanian population, 0.035; all persons with the mutation were heterozygotes. The mutation was absent in the populations from China and Surinam. This variant has been recorded in the dbSNP database, as rs16910526.

A Human Genome Diversity Project selection browser was used to obtain further information about the frequency and world distribution of the SNP; this analysis confirmed our findings (Figure 3). We analyzed haplotype diversity within a white population and a black population by studying genomewide SNP data sets for samples obtained from 1422 whites from the U.K. 1958 birth cohort [14] and 171 African Maasai from Kinyawa, Kenya (from HapMap Phase III) [15]. Allele frequencies of the polymorphism within both populations did not differ significantly from those reported previously (whites, 0.078, and blacks, 0.023, as measured in our populations) [14]. Minimal mutation-network analysis revealed that, among the 23 haplotypes found in both populations, only 2 contain the stop mutation (Figure SB in the Supplementary Appendix). Both haplotypes are present in both the European and African populations, and therefore are not population specific. In addition, the haplotypes are located near the ancient haplotype in the network, indicating that they are closely related to the ancient haplotype (Figure S5C in the Supplementary Appendix).

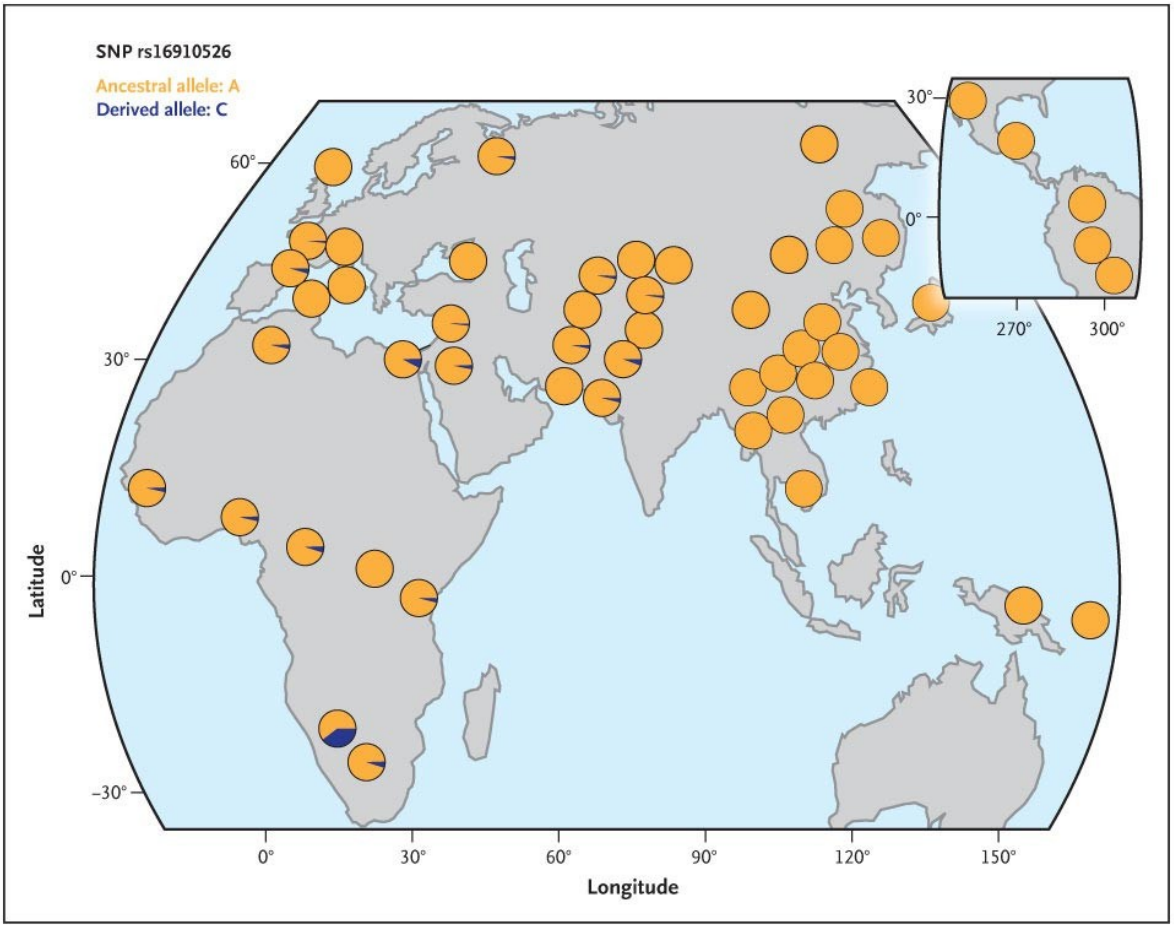

Figure 3. Polymorphisms and haplotypes of the dectin-1 stop mutation worldwide. The frequency of the dectin-1 polymorphism rs16910526 is shown for various populations of the world. The ancestral allele A leads to a functional dectin-1 receptor, whereas the derived allele C leads to an early-stop-codon mutation and nonfunctional dectin-1 receptor. The derived allele is observed only in populations from Africa and western Eurasia. The highest prevalence of the mutation (nearly 40%) is found in the San population in South Africa.

Discussion

Defective surface expression of dectin-1 due to the Tyr238X polymorphism results in lack of β-glucan recognition and an impaired cytokine response by monocytes and macrophages but normal killing of *C. albicans* by neutrophils. These data show the important role of β-glucan–dectin-1 pathways for normal activation of the cytokine response [16], but also show the redundant nature of dectin-1 in the phagocytosis and killing of *Candida albicans*. The normal function of neutrophils in persons in whom dectin-1 function is absent provides protection against invasive fungal infection. In contrast, the defective function of monocytes and macrophages with regard to cytokine release in the patients who were homozygous for the Tyr238X dectin-1 mutation is the most likely cause of the clinical phenotype characterized by mucocutaneous fungal infections.

Dysregulation of cytokine profiles is typically seen in patients with mucocutaneous fungal infections such as recurrent vulvovaginal candidiasis [17]. Dectin-1 is expressed on epithelial cells, and proinflammatory cytokines have been reported to be secreted by vaginal epithelial cells [18] and to increase the natural antifungal activity of these cells [19]. It is therefore conceivable that dectin-1 function on epithelial cells would also be defective in our patients. Our data showing that a complete deficiency of dectin-1 is accompanied by mucosal fungal infections are in line with a recent study showing the susceptibility of dectin-1–knockout mice to mucosal candidiasis [20].

Dectin-1 is also important for the development of the responses of type 17 helper T cells [21], and interleukin-17 production in our patients with defective dectin-1, as compared with healthy subjects, was reduced by 50% to 80% (Figure S4A in the Supplementary Appendix). A clinical syndrome that closely resembles the syndrome in our family, with regard to fungal complications, is the hyper-IgE syndrome. Patients with the hyper-IgE syndrome have a defect in interleukin-17 production in response to infections with *Staphylococcus aureus* (cutaneous and pulmonary infection) and *C. albicans* (onychomycosis and mucosal infection) [22,23]. Our family had an isolated interleukin-17 defect in response to *C. albicans* stimulation, and it is tempting to speculate that this is the cause of the clinical picture of fungal infections in the patients. Our findings are strengthened by the description in this issue of the Journal of a family with a mutation of CARD9, the adaptor molecule for dectin-1, who presented with a phenotype practically identical to that of our family [24].

Finally, the finding of this dectin-1 mutation in persons from both Europe and Africa leads to additional hypotheses. First, the identification of this polymorphism in all African populations assessed (including the San population) suggests that this is an ancient mutation that most likely emerged more than 60,000 years ago, before the split of the modern human populations in the late Paleolithic [25]. This hypothesis is supported by the finding that the location of the haplotypes containing the Tyr238X mutation was close to the ancient haplotype. Second, the relatively high prevalence of the polymorphism in these populations may represent an important genetic susceptibility factor for mucosal fungal infection.

Materials and methods

Index family and other subjects

To identify functional defects in the immune response to *C. albicans*, we designed a functional screening assay of mononuclear cells isolated from patients with recurrent vulvovaginal candidiasis or oral or esophageal candidiasis. Cells were stimulated with heat-killed *C. albicans*, and cytokine production after 24 hours was assessed during screening. An immunologic and genetic analysis was performed on mononuclear cells obtained from a patient who had defective cytokine production, on stimulation with *C. albicans* or β-glucan, and from four family members. The study protocol was approved by the ethics committee of Nijmegen–Arnhem, the Netherlands. Written informed consent was obtained from all family members studied.

In addition, we studied DNA samples, previously collected to assess the prevalence of the Tyr238X mutations, from several populations in various geographic locations. These samples were obtained from the country of origin, with the exception of the Chinese Han cohort, for which samples were from the Corriell Institute (catalog number, HD1000CHI). For all populations, subjects had provided written informed consent, and study approval was obtained from local ethics committees.

For haplotype analyses, we used genomewide data on single-nucleotide polymorphisms (SNPs) from 1422 whites from the 1958 United Kingdom birth cohort [14] and from 171 Maasai from Kinyawa, Kenya [15]. Genotype data for the Maasai population were downloaded from the International HapMap Project Web site (www.hapmap.org) and for the white population were obtained with the use of HumanHap300 BeadChips (Illumina). A region of 20 kb around the SNP rs16910526 was extracted from all samples in both genomewide SNP data sets. We used the Beagle software program to determine the haplotypes from genotypes of all subjects [26].

Genetic analysis

A detailed description of the methods used to sequence the dectin-1 gene is presented in the Supplementary Appendix (available with the full text of this article at NEJM.org). Network analysis was performed with the Network program, version 4.5.0.0 (www.fluxus-engineering.com), which uses the median-joining method [27]. The ancient haplotype was determined with the use of the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/). Various analyses were conducted, with weighting applied separately for synonymous changes, nonsynonymous changes, transitions, and transversions.

Modeling of dectin-1 structure

The crystal structure of mouse dectin (Protein Data Bank code 2cl8 [PDB]) was used as a template to build a homology model of the extracellular domain of human dectin-1. Modeling of the extracellular domain was performed on the "WHAT IF" server

(http.//swift.cmbi.ru.nl). Energy minimization and analysis were performed, to obtain the most reliable model of protein folding, with the YASARA program (www.yasara.org/) [28].

Fluorescence-Activated Cell Sorting

Human peripheral-blood mononuclear cells (PBMCs) were obtained from the three family members who were homozygous for a dectin-1 mutation, the two family members who were heterozygous for a dectin-1 mutation, and five persons who were homozygous for the wild-type dectin-1 allele. The cells were incubated with murine anti–dectin-1 monoclonal antibody GE2 (5 µg per milliliter) [29] or isotype-control antibody, followed by allophycocyanin-conjugated goat antimouse antibody (Pharmingen). Dectin-1 expression was determined by means of flow cytometry with the use of a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences). Dectin-1 expression was assessed in cells from a mouse embryonic fibroblast cell line (NIH3T3) after transfection, with the use of an antihemagglutinin antibody (Covance), an IgG1 isotype-control antibody, and an antimouse antibody covalently labeled with R-phycoerythrin (Jackson ImmunoResearch).

Candida binding, phagocytosis, and fungal-killing assays

Binding of PBMCs to *C. albicans* yeast cells was measured by means of flow cytometry [30]. The phagocytosis and fungal-killing assays for *C. albicans* were performed as previously described [31].

Cytokine measurements

PBMCs [12] ($5x10^5$) were incubated with 100 µl of β-glucan (10 µg per milliliter) and *C. albicans* that was live or had been heat-killed (through heating for 30 minutes at 56°C) at a concentration of $1x10^6$ yeast cells per milliliter. For interleukin-17 stimulation assays, PBMCs were stimulated for 5 days in RPMI medium supplemented with 10% pooled human serum. After 4 hours, 24 hours, or 5 days of incubation at 37°C, supernatants were collected and stored at –70°C until enzyme-linked immunosorbent assay with the PeliKine-Tool set of reagents (Sanquin).

Cloning and transfection studies

The human dectin-1 isoforms were amplified, by means of a polymerase-chain-reaction (PCR) assay, from complementary DNA isolated from samples of peripheral-blood leukocytes obtained from the patients. The two primers used were 5'-AAAGGATCCAGGGGCTCTCAAGAACAATG-3' and 5'-AAACTCGAGTCTTCCACCCTTCCCCTTAC-3'. The PCR products were purified with the use of the QIAquick PCR purification kit (Qiagen), cloned into the pCR4-TOPO3.1 vector (Invitrogen), and sequenced. The wild-type and mutant dectin-1 were cloned with the use of reverse primers — 5'-CCCTTCCTCGAGCATTGAAAACTTC-3' and 5'-AAATCTCGAGTGAGGGCAGACTAC-3', respectively — allowing for the in-frame cloning of a hemagglutinin tag, which does not affect β-glucan binding [7]. The products were subcloned into the retroviral vector pFB-Neo (Stratagene) and transfected into ecotropic packaging cells (Phoenix) with the use of FuGENE transfection reagent (Roche Molecular Biochemicals). After 48 hours, retroviral supernatants were

harvested and used to transduce NIH3T3 cells in the presence of Polybrene (Sigma), at a concentration of 5 μg per milliliter. A binding assay for zymosan was also performed, as previously described [32].

Acknowledgements

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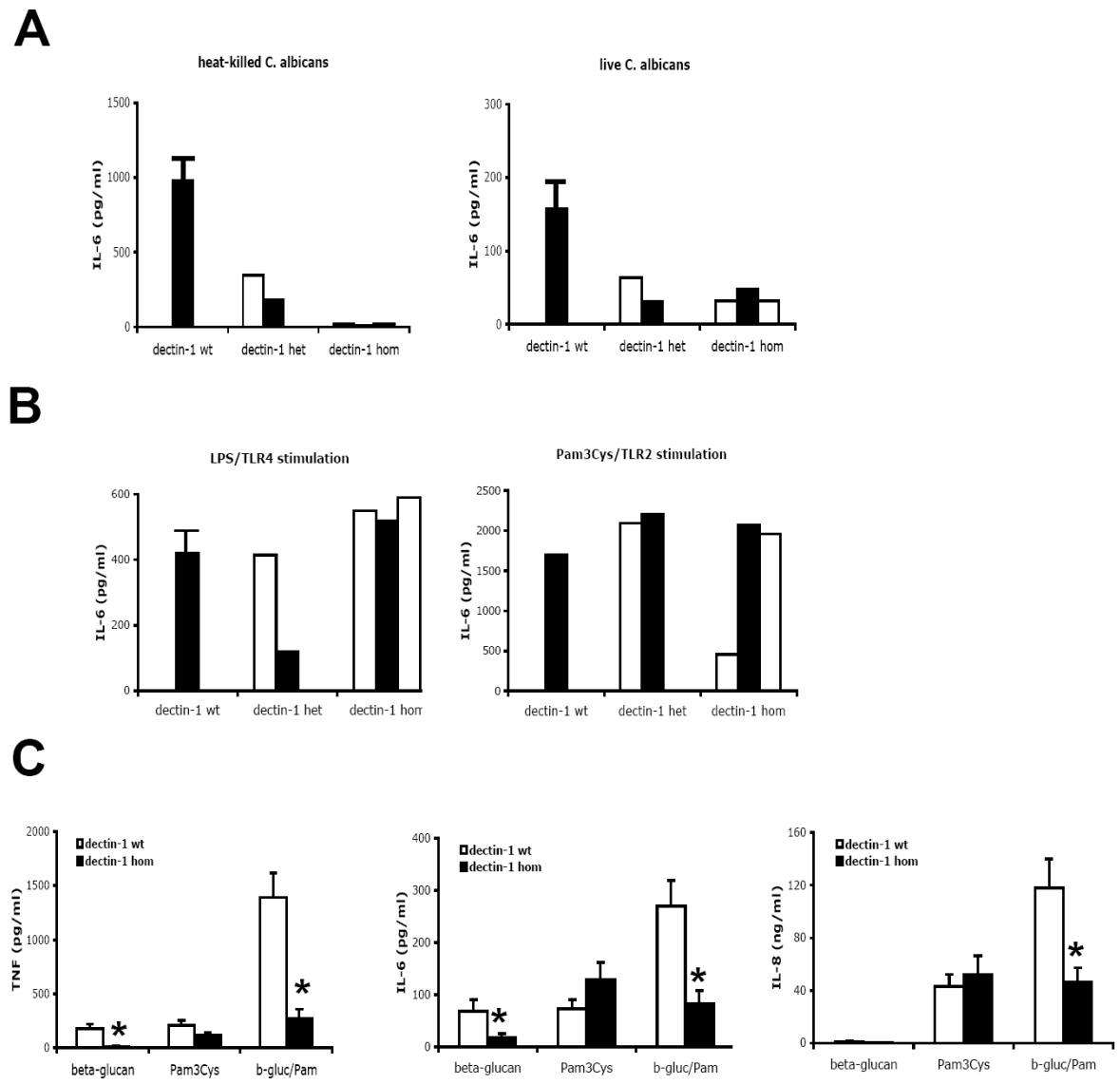
Supplemental methods and figures

Isolation of cDNA, DNA and development of primers

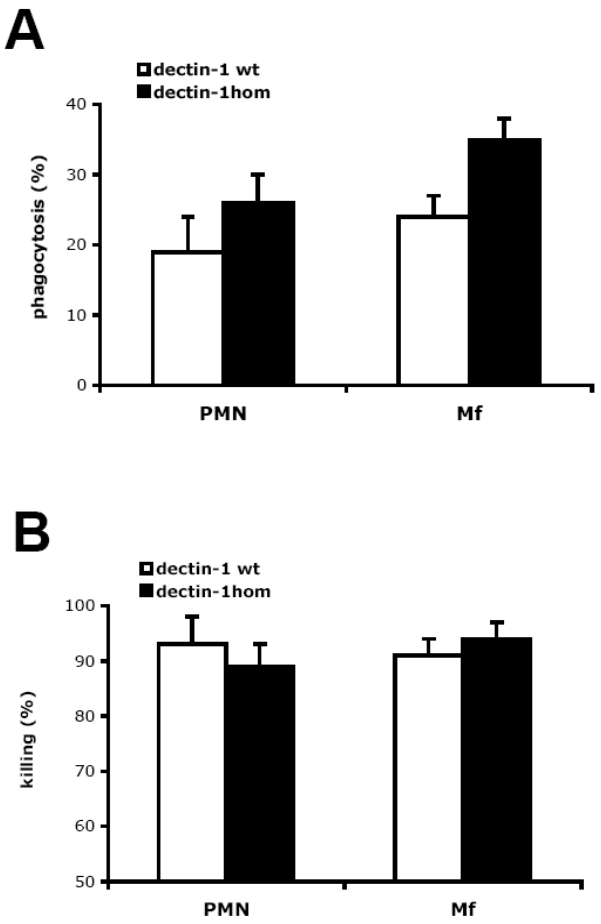
RNA was isolated from whole blood using the PAXgene blood RNA kit (Qiagen) according to the protocol. Isolated RNA was thereafter treated with DNase (Ambion) and reverse transcribed to cDNA using oligo(dT) primers and MMLV reverse transcriptase (Invitrogen). PCR on the cDNA to investigate transcription of the different Dectin-1 isoforms was performed with primer pairs adapted from Meyer- Wentrup et al (2007). DNA isolation was performed on whole blood using the isolation kit Puregenetm (Gentra system). DNA template of the Dectin-1 (also named CLEC-7A) sequence was taken from Genebank, chromosome position 12q13, NC_000012.10 (10160643..10174135, complement). Primer3 software was used to develop all primers shown in Table 1. After amplification PCR products were cleaned using the kit High Pure PCR product purification kit (Roche) according to the manufacture protocol. Sequencing was performed at the Sequence Faculty at the department of human genetics Nijmegen. Sequence for four out of six exons was obtained using both forward and reverse primers (Table S1). However, equences for exon 3 and 4 were based on more than one forward sequences due to the lack of reverse primer specificity. No variations were detected in any exons other than exon 6.

Exon	Primer	Sequence (5Õ to 3Õ)	[$MgCl_2$] (mM)	Annealing temperature
1	Dectin-1_205_FW	TTT-CAC-CAC-GTT-AGC-CAA-GCT	2.5	52 ^{0}C
	Dectin-1_205_RV	CTG-AAA-TAG-TTT-GCA-TCG-GTT		
2	Dectin-1_203_FW	CCC-TTT-ATA-AGT-GAA-ATG-GGC	1.75	60 ^{0}C
	Dectin-1_203_RV	ACC-GTG-CAA-GGC-CAG-ATT-TT		
3	Dectin-1_127_FW	GCC-AGT-GAT-AAA-TCA-GTT-ACT	3.5	56^{0}C
	Dectin-1_127_RV	TTC-TTC-TTC-TCC-ACC-TTC-TT		
3	Dectin-1_202_FW	TGG-CAA-CAT-TTT-CCC-TTC-TT	3.5	56 ^{0}C
	Dectin-1_202_RV	GCC-AAG-GGC-ATA-GTT-AAA-GG		
4	Dectin-1_301_FW	TCA-TTA-CCT-GGA-ATC-TCC-CTC-T	2.5	56 ^{0}C
	Dectin-1_301_RV	TGG-CAA-CTA-ATT-GGT-TAT-TTC-A		
5	Dectin-1_119_FW	GCT-GCT-CGA-CAG-AGG-TTT-TC	1.75	62 ^{0}C
	Dectin-1_119_RV	GGA-TGG-TCT-CGA-TCT-CCT-GA		
6	Dectin-1_106_FW	AAT-CAC-AGC-CTC-TCC-CTT-CA	2.5	60 ^{0}C
	Dectin-1_106_RV	GAT-TTA-AGC-CTC-CTT-TTC-CAA		

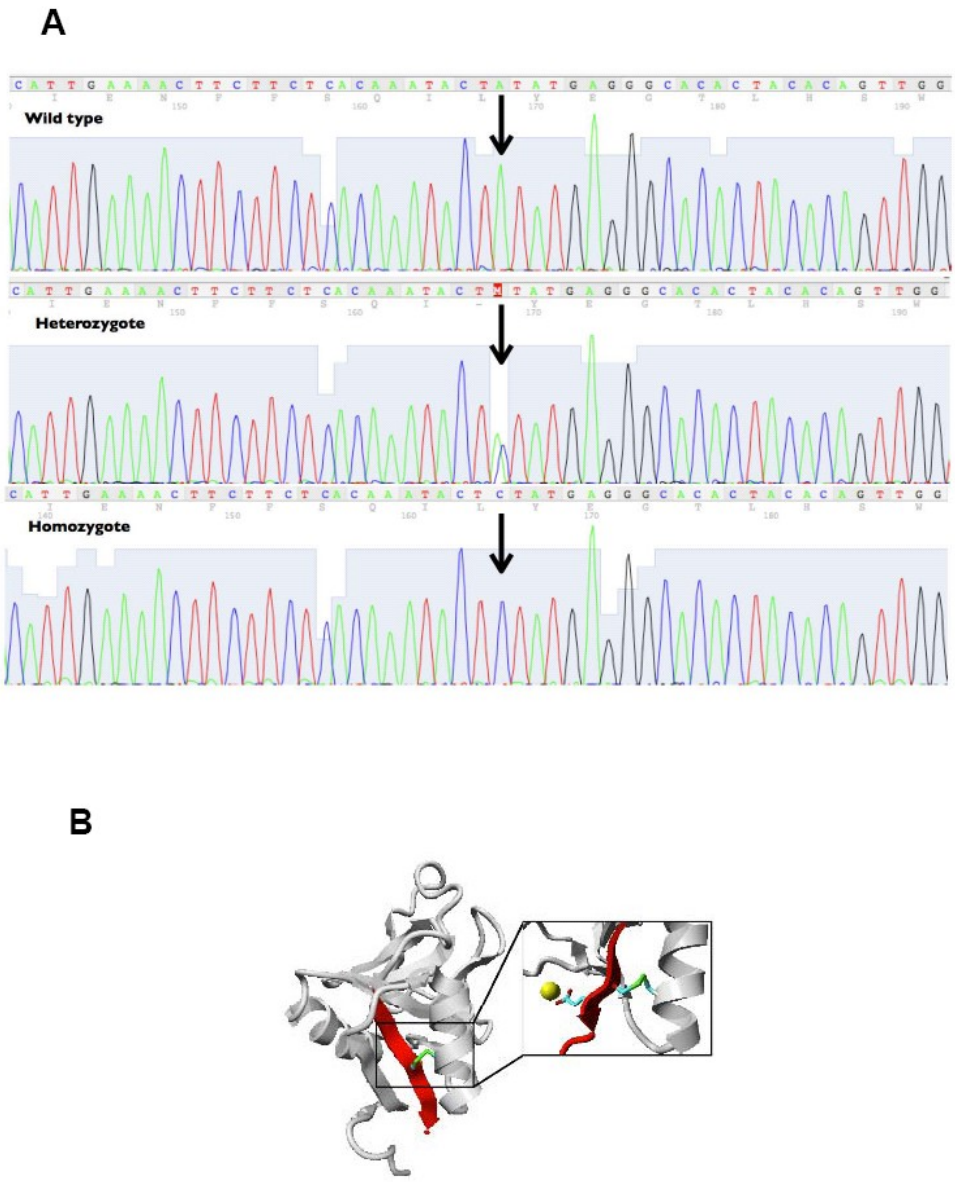
Supplementary table S1



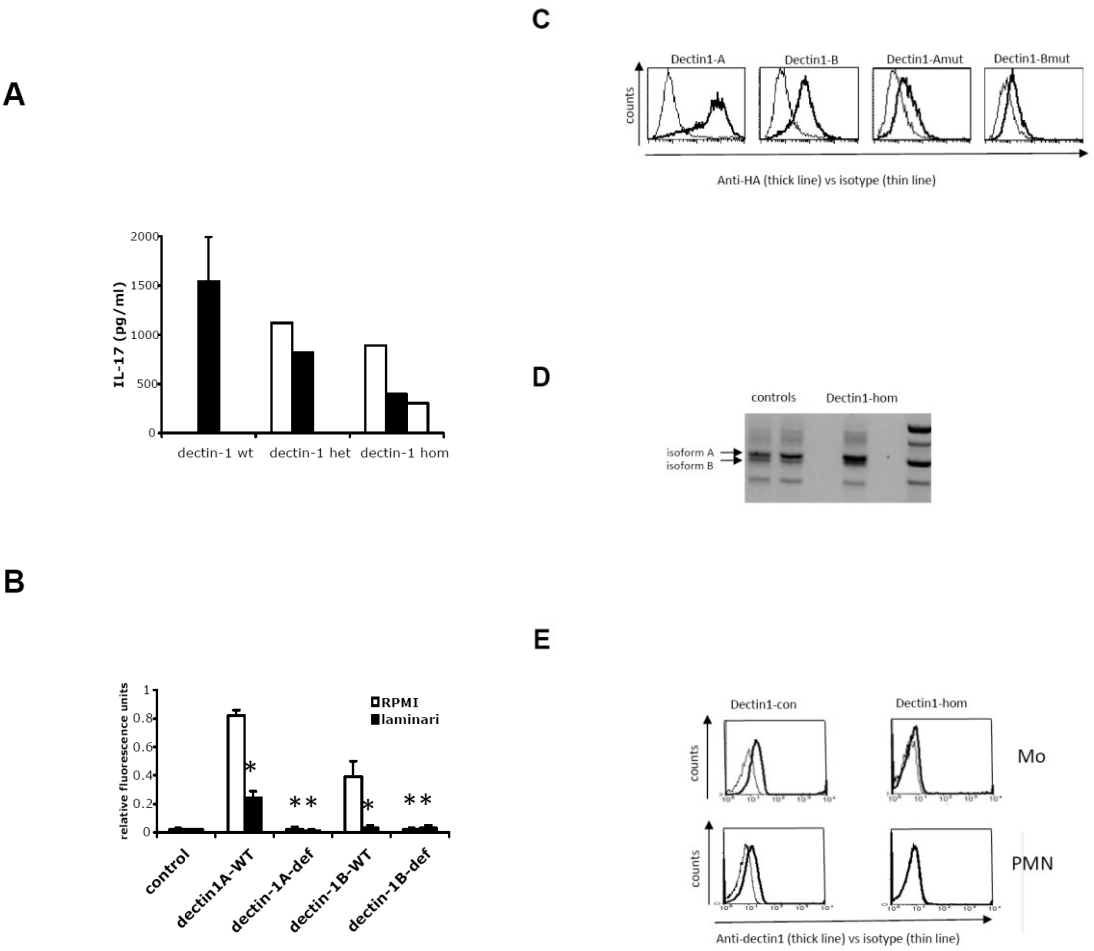
Supplementary figure S1. Dectin-1 mutation leads to functional defects. **Panel A:** IL-6 production capacity of monocytes isolated from healthy volunteers (dectin-1 wt), patients heterozygous (dectin-1 het), or patients homozygous for the mutation (dectin-1 hom), and stimulated for 4h with heat-killed or live *C. albicans*. Each patient represents a white or a black bar. Variation in the control group is presented as SD (n=5). **Panel B**: IL-6 production capacity of monocytes isolated from healthy volunteers (dectin-1 wt), patients heterozygous (dectin-1 het), or patients homozygous for the mutation (dectin-1 hom), and stimulated for 4h with the TLR4 antagonist LPS, or the TLR2 antagonist Pam3Cys. **Panel C:** Synergism between dectin-1 and TLR2 stimulation of proinflammatory cytokines in cells isolated from control volunteers (dectin-1 wt) or patients homozygous (dectin-1 hom) for the stop codon mutation, after 24h stimulation at 37°C. (means ± SD of three experiments, n=5 healthy volunteers, n=3 homozygous patients; $p<0.05$).



Supplementary figure S2. Dectin-1 deficiency does not impair *Candida* phagocytosis and killing. Phagocytosis (A) and killing (B) of opsonized *C. albicans* by monocytes and neutrophils from healthy volunteers (five healthy volunteers without clinical signs of fungal infections, dectin-1 con) or patients homozygous for the mutation (dectin-1 hom). Using this methodology in medium enriched with 10% human serum, phagocytosis receptors such as Fc-receptors or complement-mediated binding are apparently sufficient to compensate for the absence of dectin-1. Results are presented as means ± SD of four experiments.

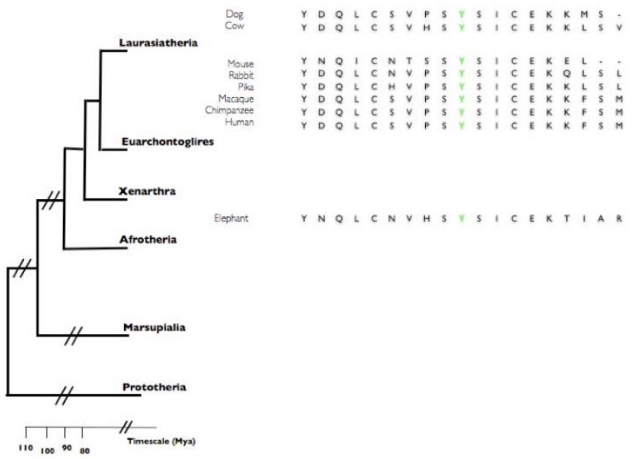


Supplementary figure S3. Dectin-1 mutation in a family with mucocutaneous fungal infections. **Panel A:** The nucleotide change (A > C) in exon 6 is shown in a person with the functional dectin-1 receptor (wild type) and two individuals having the stop mutation in heterozygote or homozygote state. **Panel B:** Modeling of the mutated dectin-1 receptor. The missing nine aminoacids are depicted in red, the diagram showing the loss of a disulfide bridge and a Ca^{2+}-binding site.



Supplementary figure S4. Dectin-1 mutation leads to functional defects. **Panel A:** IL-17 production capacity of PBMC from healthy volunteers (dectin-1 wt), patients heterozygous (dectin-1 het), or patients homozygous for the mutation (dectin-1 hom) stimulated for 5 days with heat-killed C. albicans. **Panel B:** Only wild-type, but not mutated, isoforms A or B of dectin-1 were able to mediate binding of zymosan to the transfected NIH3T3 cells. The specificity of the binding was demonstrated by its inhibition with the dectin-1 antagonist laminarin. Representative results of five independent experiments are presented. **Panel C:** The variant dectin-1 isoforms A (dectin-1Amut) and isoform B (dectin1-Bmut) were poorly expressed on the surface of transfected NIHT3T cells, when compared to wild-type dectin-1 isoforms. **Panel D:** The pattern of transcription of the dectin-1 isoforms is similar between healthy individuals with wild-type dectin-1 gene and patients homozygous for the early stop codon mutation. **Panel E:** Extracellular FACS staining of monocytes (Mo) and neutrophils (PMN) isolated from healthy volunteers (dectin-1 con) or patients homozygous for the mutation (dectin-1 hom).

A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Cow Y D Q L C S V H S Y S I C E K K L S V
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Mouse Y N Q I C N T S S Y S I C E K E L - -
Rabbit Y D Q L C N V P S Y S I C E K Q L S L
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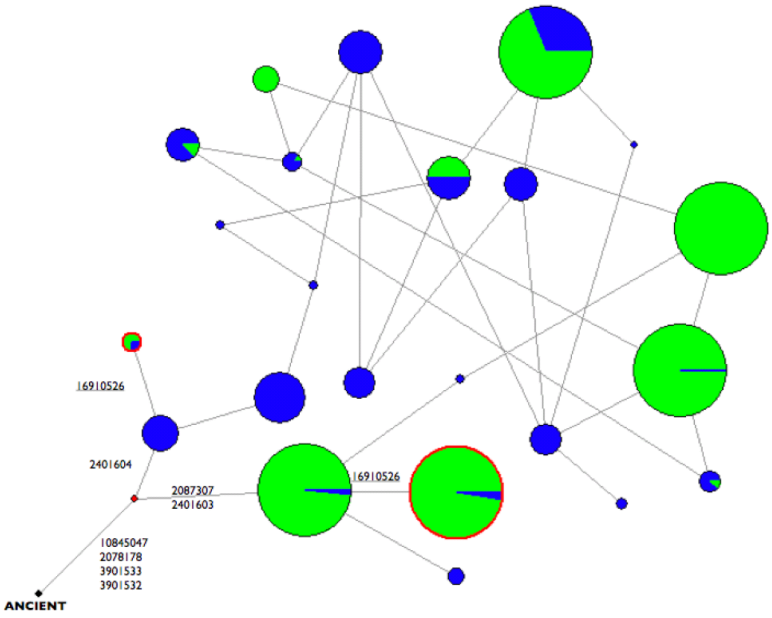
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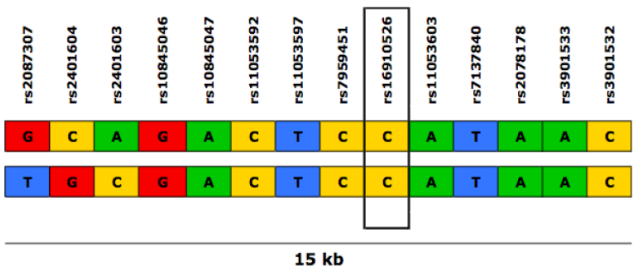
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Supplementary Figure S5. Dectin-1 stop mutation/polymorphism and haplotypes in different populations. **Panel A:** Phylogenetic representation of the dectin-1 receptor aminoacid area surrounding the Tyr238Stop, shown in green, in the mammalian lineage showing that the location of the mutation is conserved. **Panel B:** Dectin-1 allele haplotype network of 1422 Caucasian (2844 haplotypes) and 171 African (342 haplotypes) individuals. Each circle represents a unique haplotype node and colors represent the different populations. Green is used for Caucasian haplotypes, Blue for African, and black for the ancient haplotype according to the dpSNP information. Circles with red outline contain the two haplotypes containing the stop polymorphism. The red circle following the ancient haplotype represents a vector node and does not represent an observed haplotype. Area of all circles indicates the amount of alleles with that haplotype. At the lower part, which includes the two haplotypes containing the stop polymorphism, SNP rs numbers that change between the nods are presented. **Panel C:**The two haplotypes containing the 238Stop mutations. Only the first three SNPs differ between these haplotypes, which are located after exon 6.

Reference List

1 Sobel,J.D. (2007) Vulvovaginal candidosis. *Lancet* 369, 1961-1971

2 Scher,R.K. *et al.* (2007) Onychomycosis: diagnosis and definition of cure. *J. Am. Acad. Dermatol.* 56, 939-944

3 Spellberg,B.J. *et al.* (2006) Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* 42, 244-251

4 Netea,M.G. *et al.* (2008) An integrated model of the recognition of Candida albicans by the innate immune system. *Nat. Rev. Microbiol.* 6, 67-78

5 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

6 Jouault,T. *et al.* (2003) Candida albicans phospholipomannan is sensed through toll-like receptors. *J. Infect. Dis.* 188, 165-172

7 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

8 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

9 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

10 Saijo,S. *et al.* (2007) Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat. Immunol.* 8, 39-46

11 Dennehy,K.M. *et al.* (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* 38, 500-506

12 Gow,N.A. *et al.* (2007) Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* 196, 1565-1571

13 Gantner,B.N. *et al.* (2005) Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *EMBO J.* 24, 1277-1286

14 van Heel,D.A. *et al.* (2007) A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat. Genet.* 39, 827-829

15 Frazer,K.A. *et al.* (2007) A second generation human haplotype map of over 3.1 million SNPs. *Nature* 449, 851-861

16 Brown,G.D. (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 6, 33-43

17 Babula,O. *et al.* (2005) Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.* 40, 1258-1262

18 Steele,C. and Fidel,P.L., Jr. (2002) Cytokine and chemokine production by human oral and vaginal epithelial cells in response to Candida albicans. *Infect. Immun.* 70, 577-583

19 Barousse,M.M. *et al.* (2001) Growth inhibition of Candida albicans by human vaginal epithelial cells. *J. Infect. Dis.* 184, 1489-1493

20 Hise,A.G. *et al.* (2009) An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. *Cell Host. Microbe* 5, 487-497

21 LeibundGut-Landmann,S. *et al.* (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8, 630-638

22 Grimbacher,B. *et al.* (1999) Hyper-IgE syndrome with recurrent infections--an autosomal dominant multisystem disorder. *N. Engl. J. Med.* 340, 692-702

23 Milner,J.D. *et al.* (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452, 773-776

24 Glocker,E.O. *et al.* (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N. Engl. J. Med.* 361, 1727-1735

25 Underhill,P.A. *et al.* (2000) Y chromosome sequence variation and the history of human populations. *Nat. Genet.* 26, 358-361

26 Browning,S.R. and Browning,B.L. (2007) Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. *Am. J. Hum. Genet.* 81, 1084-1097

27 Bandelt,H.J. *et al.* (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, 37-48

28 Krieger,E. *et al.* (2002) Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. *Proteins* 47, 393-402

29 Willment,J.A. *et al.* (2005) The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells. *Eur. J. Immunol.* 35, 1539-1547

30 Cambi,A. *et al.* (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *Eur. J. Immunol.* 33, 532-538

31 Vonk,A.G. *et al.* (2002) Phagocytosis and intracellular killing of Candida albicans blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *J. Microbiol. Methods* 49, 55-62

32 Willment,J.A. *et al.* (2001) Characterization of the human beta -glucan receptor and its alternatively spliced isoforms. *J. Biol. Chem.* 276, 43818-43823

Chapter 6

The role of Toll-like receptors and C-type lectins for vaccination against *Candida albicans*

Gerben Ferwerda, Mihai G. Netea, Leo A. Joosten, Jos W.M. van der Meer, Luigina Romani and Bart Jan Kullberg

Vaccine. 2009 Oct 31

Review

The incidence of invasive fungal infections has increased over the past decades, due to the augmented use of immunosuppressive therapy, higher incidence of HIV, performance of major surgical procedures and aging of the population [1-3]. *Candida* species are the fourth most common cause of nosocomial bloodstream infections and have a mortality rate up to 40% despite the availability of new effective antifungal agents [4,5]. The current consensus is that antifungal treatments strategies supplemented with immunotherapy, or vaccination in specific patient groups at risk, can improve the outcome and reduce mortality [6,7].

The role of innate and acquired immunity for vaccination

Long-lasting protection against microorganisms is gained through the activation of the adaptive immune system represented by cellular immune and humoral immune responses [8]. Depending on type of pathogen and site of infection, an adjusted balance between cellular and humoral responses is desirable [9,10]. Historically, development of many vaccines emphasized induction of the humoral response. However, vaccines against pathogens that cause chronic or mucosal infections should preferable elicit a cellular response [11]. Attenuated or killed microorganisms and pathogen specific proteins or polysaccharide-protein conjugates have been used as vaccination strategies [8]. Three signals are critical for effective stimulation of an adaptive immune response: (a) the presentation of antigens by antigen-presenting cells (APC) on MHC-II to T-cell receptor (TCR) on naïve CD4+ T-cell, (b) the up-regulation of co-stimulatory molecules, and (c) production of cytokines that direct differentiation of a certain type of helper T-cell (Th) response (Figure 1). This process is induced and regulated by recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the surface of APCs [12-14]. Depending on the cytokine cocktails induced by PRR activation, the induction of the adaptive immune response is directed towards a Th1, Th2 or Th17 phenotype [15-17]. Optimal inflammatory conditions are induced by immunization with living organisms, while vaccines containing only purified antigens need addition of adjuvants that have to induce stimulation of co-stimulatory molecules and cytokines [11,18]. Most commonly used adjuvants are aluminum and calcium salts which are licensed for human use and enhance the humoral response [19] and they induce their effects through activation of PRRs. For example alum has been shown to exert its effect through recognition by NALP3, a pattern recognition receptor from the NLR family [20,21]. It is also expected that the novel squalene-based emulsion MF59 acts through activation of intracellular innate immune receptors, although the precise mechanism has to be further investigated [22,23]. The deeper insight in the innate immune system obtained in the last years has lead to the design of new adjuvants based on PAMP-PRR interaction, in order to enhance specific cellular immunity elicited by vaccines. One such adjuvant now in clinical trials is the TLR9 agonist CpG-DNA [24].

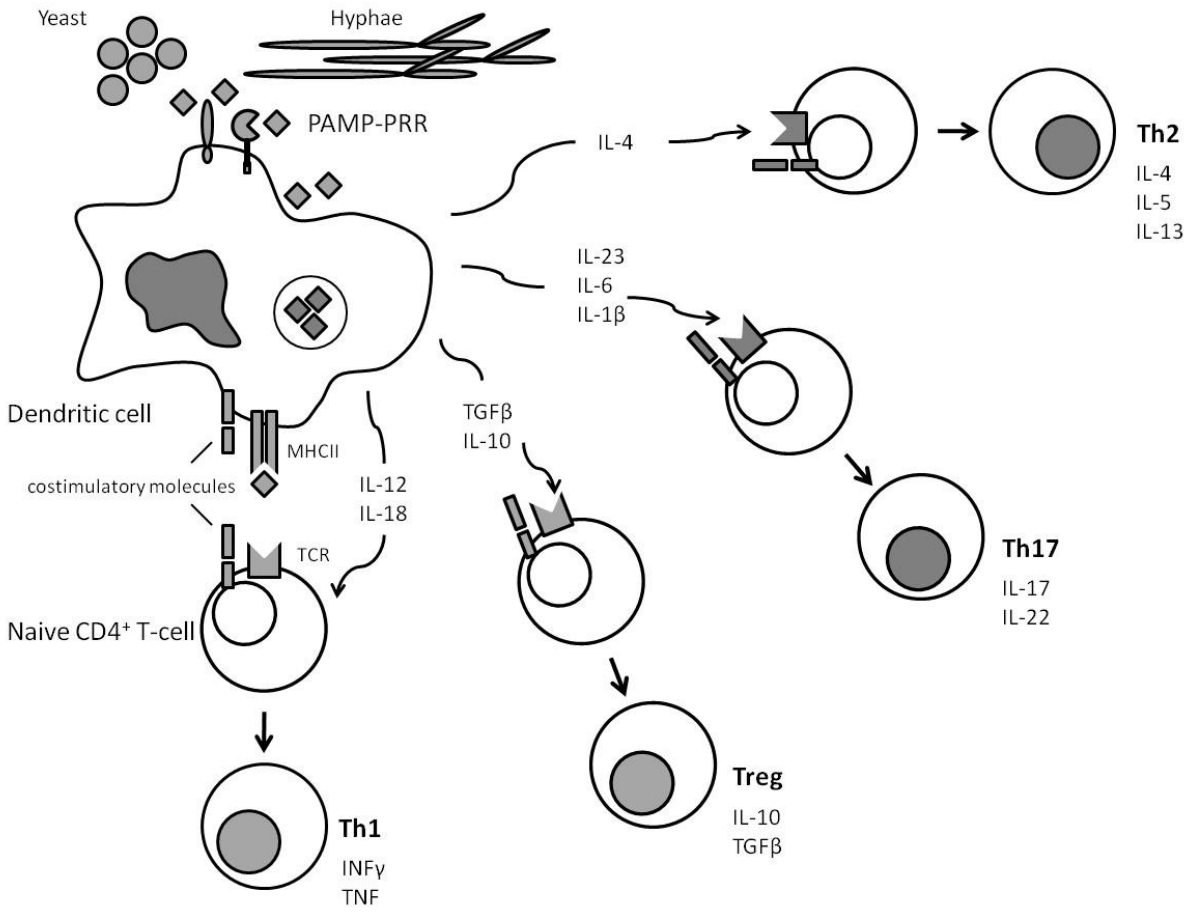


Figure 1. Modulation of T-cell responses by innate immune system. Engagement of pathogen associated molecular patterns (PAMPs) from *Candida albicans* by pattern recognition receptors (PRRs) lead to the induction of specific cytokine profiles regulating the differentiation of naive $CD4^+$ T-cells in to Th1 (T-helper 1), Th2 (T-helper 2), Th17 (IL-17 secreting T-helper) or Treg (T-regulatory) cells. For activating naïve CD4+ T-cells, presentation of antigens on MHC class II to T-cell receptor (TCR) is necessary in combination with engagement of co-stimulatory molecules.

Vaccination strategy against Candida albicans

C. albicans is a commensal microorganism, capable of colonizing mucosal sites of humans. Normally this does not lead to disease, because the epithelial barrier protects us against invasion of fungi. This protection is based on the anatomical barrier and the local immune response, both limiting the fungal burden and leading to a state of tolerance. When the local immune response is insufficient, a high fungal burden at the mucosal site will cause mucocutaneous candidiasis. When the epithelial barrier is breached, for example by surgical procedures or catheters, the local immune response will be crucial to prevent translocation of fungi to the blood stream (candidemia), which can lead to systemic candidiasis when the fungi cannot be cleared. Individuals with an impaired mucosal immune response have and a high local fungal burden and are at higher risk to develop a systemic candidiasis when their epithelial barrier is breached, or their systemic immune response is insufficient (e.g. neutropenia).

The innate host defense and cellular immune responses against *C. albicans* play an important role in the pathogenesis of mucocutaneous candidiasis, as illustrated by the high incidence of *C. albicans* infections in patients with HIV infection and low number of CD4+ T-cells [25,26], or after treatment with immunosuppressive agents like corticosteroids [27]. These clinical facts are strengthened by results obtained in experimental models of candidiasis in which it has been shown that the T-cells lead to protection against mucosal [28,29] and disseminated [30] *C. albicans* infections. The humoral immune response may also play a role in invasive candidiasis, as shown by the antibody response against specific fungal cell-wall components in *C. albicans* infections [31,32]. Opsonization by antibodies leads to enhanced phagocytosis and killing of *C. albicans* by human and murine mononuclear cells *in vitro* [33-35], while B-cell knock-out mice are more susceptible to experimental systemic, but not mucosal, candidiasis [36]. Interestingly however, there is no higher incidence of fungal infections in human with immunoglobulin disorders [37,38]. Passive immunization and the humoral response after active vaccination induces protection against disseminated infections with *C. albicans* [39,40], but does not seems to play a crucial role in mucosal infections [41]. At this moment there is no active *Candida* vaccine available for humans, and passive immunization with neutralizing or opsonizing antibodies are in clinical trials. An excellent overview of the progress in development of fungal vaccines has recently published [7], but less work has been done regarding the optimal adjuvants to be used for *C. albicans* vaccines.
Different strategies have been used to develop an active vaccine against *C. albicans*. The use of specific cell-wall components of *Candida* like Als1P, Als3P, Hsp90, mannans or enzymes like SAP2 [42-45], led to protection against systemic *C. albicans* infection in mice as measured by decreased fungal outgrowth and improved survival. This response was mainly mediated by cellular immunity [30]. These vaccines were mostly administered subcutaneously (s.c.) and adjuvants were needed to optimize protection. In other vaccination models, primary administration of a low dose of live *C. albicans* [46] or a low virulent *Candida* strain like PCA2 [47,48] intravenously (i.v.) led to protection after reinfection with a high dose of *C. albicans*. Orally administered or locally administered *C. albicans* in a low inoculum protected mice against reinfection with a higher infectious inoculum, this process was mediated by cellular immune mechanisms [41]. These models provided insight in the type of cellular response needed for protection against *Candida* and provided a proof-of-principle that vaccination against *Candida* is feasible.

Type of cellular responses needed for protection against Candida infections

Activation of the innate immune system by *C. albicans* induces the production of a variety of proinflammatory cytokines and the expression of co-stimulatory molecules, which in turn mediate specific adaptive immune responses leading to protection against disseminated candidiasis and tolerance at the mucosal barrier. It is generally accepted that induction of a Th1 type cellular response is crucial for the defense against *C. albicans* [49-51]. In contrast, a Th2 cellular response is considered non-protective, as it induces class-switch to non-opsonizing antibody subclasses and IgE [52]. Investigation of the role of Th17 in mediating the immune response has shown that Th17 memory cells are induced by *Candida* hyphae [53,54],

and in a murine model IL-17AR knock-out mice had an increased susceptibility to systemic candidiasis [55]. However, deleterious effects of IL-17 inflammatory activities have also been described [56-58]. Patients with an impaired IL-17 production suffer from mucosal *C. albicans* infections in hyper IgE syndrome (HIES) and chronic mucocutaneous candidiasis (CMC) [59-61]. In contrast to T-helper cells, regulatory T-cells (Treg) suppress inflammatory responses in disseminated *C. albicans,* resulting in higher susceptibility in mice [62,63]. However, the tolerization-inducing effects of Tregs seem to be beneficial at mucosal sites [58,64]. Summarizing these data, one could conclude that for optimal protection against (chronic) mucosal *Candida* infections Th1, Th17 and Treg are important. An effective Th1 and antibody (humoral) response are important for defense against disseminated *Candida* infections. This information should be considered when making a choice of an adjuvant for anti-*Candida* vaccination.

Role of TLRs in immune response against C. albicans

The PRRs involved in the recognition of *C. albicans* have been recently reviewed [65]. A short overview is given to determine the potential role of TLR ligation in modulating adaptive cellular immune responses in *Candida* vaccination.
Toll-like receptors (TLRs) are type I integral membrane glycoproteins with a cytoplasmic domain containing a signal motif homologue to interleukin-1 receptors (Toll-IL-1 receptor domain or TIR). The TIR domain can associate with adaptor molecules for downstream signaling. All TLRs use the adaptor molecule MyD88 (myeloid differentiation factor 88 adaptor molecule) for induction of intracellular signals with the exception of TLR3. TLR4 also uses adaptor molecule TRIF (**Toll**-interleukin-1 receptor domain- containing adaptor inducing IFN) for induction of type I interferons, whereas TLR3 only uses TRIF. After ligation of TLRs, activation of a protease cascade leads to nuclear translocation of transcription factors NF-κB and IRF3, followed by transcription of cytokine and chemokine genes (Figure 2). Ligation of TLRs on DCs also induces expression of co-stimulatory molecules (maturation), and this in combination with specific cytokine profiles drives the differentiation of naïve CD4+ T-cells (Figure 1). In this way TLRs function as mediators between innate and adaptive immune responses**.** TLRs might also play a direct role in humoral responses, shown by an impaired B-cell dependent antigen-specific antibody response in MyD88 -/- mice [66,67], although other groups could not confirm these results [68,69].
Several TLRs are involved in inflammatory responses induced by *C. albicans*, of which TLR2 and TLR4 are the most studied [65]. Activation of TLR2 signal pathways in APCs by ligation of *C. albicans* cell-wall components like phospholipomannan leads to production of cytokines such as TNF, IL-1β and IL-10 [70-73], while IL-12 and IFNγ is not induced, resulting in a balance favoring a Th2 and Treg cellular response [74]. Indeed, TLR2-/- mice are more resistant to lethal disseminated candidiasis, indicating a TLR2-mediated inhibitory effect on inflammation in this model [62,63]. However, other groups have reported a higher susceptibility for disseminated candidiasis in TLR2 -/- mice [73]. These discrepancies likely depend on the difference in background of mouse used and different *Candida* strains. It would be useful to have additional information on the differences in the adaptive cellular immune responses in

these models, as this could give important information over the delicate balance between Th1/Th2/Th17 response and the consequences for fungal burden and survival. Vaccination with an i.v. low virulent *Candida* strain PCA2 in TLR2-/- mice lead to lower induction of cytokines and antibodies, but these mice were normally protected against rechallenge with a virulent *C. albicans* strain [48]. After sublethal i.v. infection with a virulent *C. albicans* strain, TLR2-/- mice were not protected against lethal i.v. hyphae reinfection [75]. These differences could be explained by differences in the induction of adaptive immune responses by the PCA2 or the virulent *Candida* strain. Both blastoconidia and hyphae can induce TLR2-dependent cytokine production, whereas blastoconidia are also potent INFγ inducers (TLR4-dependent). Hyphae fail to induce INFγ and consequently induce a Th2 response [76].

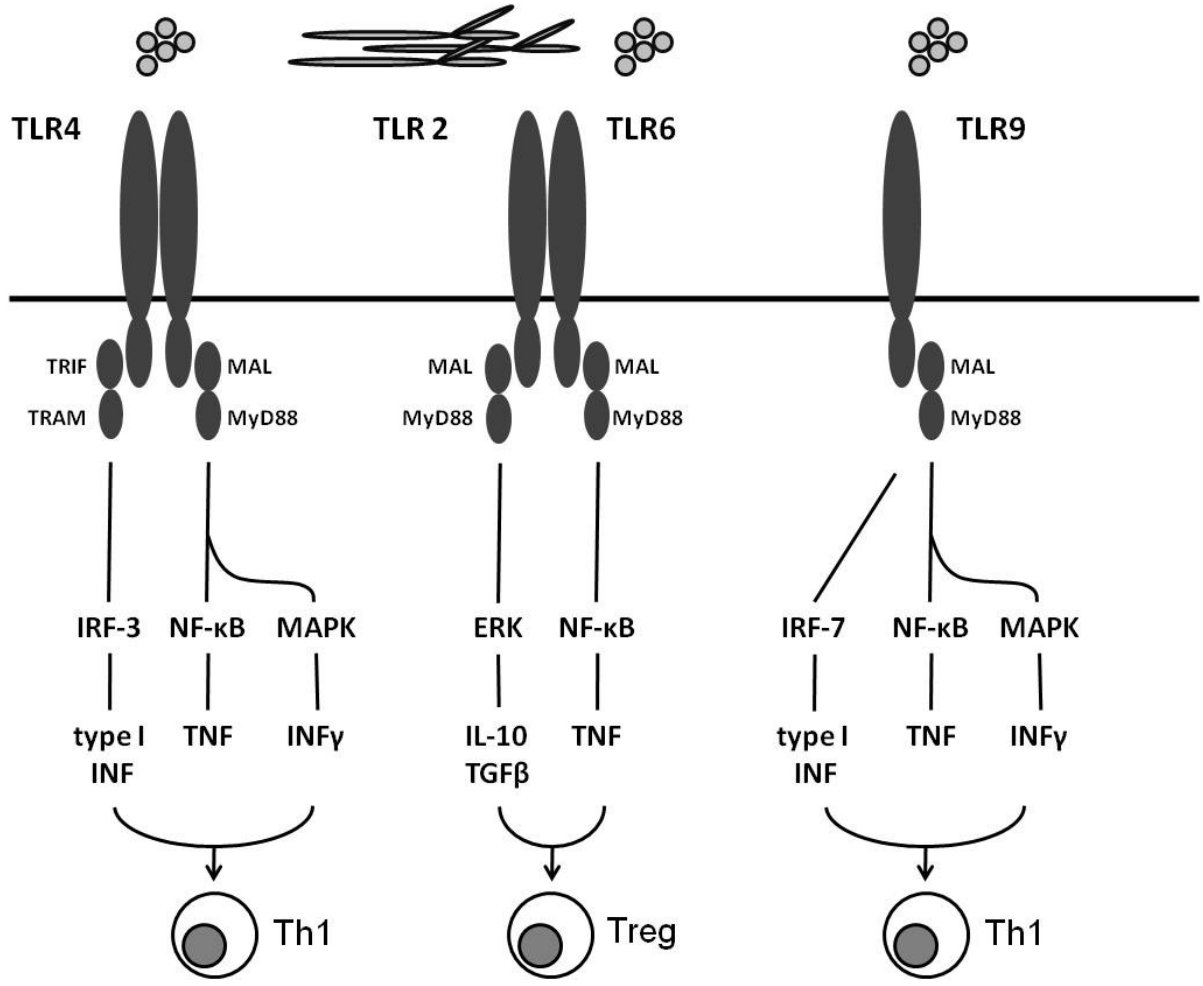


Figure 2. TLR signal pathways activated by *C. albicans* and induction of cellular immune responses. Engagement of cell-wall components and DNA of *C. albicans* conidia or hyphae by TLRs activates the Toll-IL-1 receptor domain (TIR) which lead to association with MyD88 (myeloid differentiation factor 88 adaptor molecule) or TRIF (**Toll**-interleukin-1 receptor domain- containing adaptor inducing IFN) activating a protease cascade, inducing nuclear translocation of transcription factors NF-κB and IRF3/7. Transcription of cytokine genes lead to production of specific cytokine profiles and regulation of T-cell differentiation.

TLR2 forms heterodimers with TLR1 and TLR6 [77]. While TLR1 is not involved in the recognition of *C. albicans*, TLR6 -/- showed an impaired production of IL-10 and an increased production of INFγ, similarly to TLR2-/- mice. However, the susceptibility to disseminated candidiasis was not increased in TLR6 -/-mice [78]. The effect of TLR6 has not been investigated in vaccination models.
Blastoconidia of *C. albicans* induce TLR4- mediated immune responses by ligation of O-linked mannan [79]. The role of TLR4 in disseminated candidiasis is illustrated in murine models, in which TLR4 -/- mice are more susceptible for disseminated *C. albicans* infection due to a decreased induction of KC and MIP2. This results in a deficient recruitment of neutrophils and higher fungal burden in the kidneys of TLR4 -/- mice compared to that of WT mice [71,75]. The human TLR4 polymorphisms Asp299Gly and Thr399Ile led to a higher susceptibility for *Candida* bloodstream infections [80]. Stimulation of DCs by *C. albicans* induces TLR4-dependent cytokines production like IFNγ and IL-12 resulting in a Th1-mediated cellular response [76]. Although the recognition of *C. albicans* by TLR4 has been shown in vitro and vivo, differences between various *Candida* strains may account for reports showing that TLR4 plays a minor role in candidiasis [81,82]. TLR4 also seems to be important for adaptive immune responses induced by *C. albicans*. Mice are protected against i.v. hyphal *Candida* infection after a sublethal i.v. infection with *C. albicans*, and this is mediated in a TLR4-dependent manner [75]. This inflammatory response is apparently mediated by TLR4, as O-linked mannans are known to be recognized by TLR4 [79].
Finally, TLR9 has a redundant role in disseminated candidiasis in mice, although *C. albicans* can induce TLR9-dependent cytokine production [83]. Also in a reinfection model, TLR9 -/- mice were normally protected against i.v. *C. albicans* hyphae infection after preinfection with i.v. conidia [75]. There is no data on CpG-DNA as adjuvants in *C. albicans* vaccination models, although it has been shown that they enhanced the Th1 induced protection against invasive aspergillosis in mice vaccinated with recombinant *Aspergillus* proteins [84].

Signal pathways activated by C-type lectin receptors

C-type lectins receptors (CLRs) are pattern recognition receptors (PRRs) characterized by C-type lectin-like domains (CTLDs) and some of them are involved in antifungal immunity [85]. These receptors are expressed as transmembrane proteins with or without intracellular signal motifs, or occur as extracellular proteins functioning as opsonins. Several of these CLRs can directly induce production of cytokines, while they also modulate innate and adaptive immune responses or function as binding and uptake receptors. The CLRs involved in recognition of *C. albicans* have recently been reviewed [65,86]; here we will discuss their possible role in adaptive immune responses.

Dectin-1

Dectin-1 is a type II transmembrane receptor and belongs to the NK-cell-receptor-like C-type lectin receptor family (CLRs) [87]. Dectin-1 has an extracellular carbohydrate-recognition domain (CRD), a stalk region and an intracellular immunoreceptor tyrosine-based activation motif (ITAM)-like motif [88]. Phosphorylation of the single tyrosine in this signal motif leads to

recruitment of SH2-domain-containing protein spleen tyrosine kinase (SYK) in a Scr kinase dependent manner [89,90]. Activation of SYK pathways by dectin-1 is not induced in all myeloid cells and seems to be restricted to dendritic cells and a subset of macrophages which are reversibly programmed by growth factors like GM-CSF [90,91]. Dectin-1 signaling in myeloid cells (BMDC) recruit SYK dependent CARD9 and Bcl-10-Malt1 leading to activation of transcription factors MAPK and NF-κB [92-94], resulting in cytokine production and maturation of DCs. Dectin-1 also synergizes with TLR2 and TLR4-induced signals that are dependent on the adaptor molecule MyD88, inducing TNF, IL-10, TGF-β and maturation of DCs [95-97]. Nuclear transcriptional factor NFAT is activated by dectin-1 signaling after engagement of zymosan leading to the production of cytokines independent of TLR2 [98]. The signal pathway involved in the induction of phagocytosis of β-glucan by dectin-1 in macrophages is depending on the proximal tyrosine of the ITAM-like motif and Src kinase activation, but is SYK independent [90,99]. Engagement of dectin-1 induces phagocytosis mediated by the GTPases Cdc42 and RAC-1 involving reorganization of the actin cytoskeleton [99,100].

Role of dectin-1 in the immune response against C. albicans

The cell wall of *C. albicans* consists of large amounts of β-glucan that is covered by a layer of mannans that prevent direct exposure to dectin-1. β-glucan is exposed and can bind dectin-1 only at the level of the budding scars of blastoconidia, where the integrity of the cell wall is disrupted. Hyphae do also have a thin layer of β-glucan in their cell wall which can be detected by antibodies against β-glucan [101], however, this β-glucan does not seem to bind dectin-1 [65,102]. Dectin-1-/- mice are more susceptible to disseminated candidiasis [103], and this is observation is strengthened by higher susceptibility to disseminated candidiasis in CARD9 -/- mice [92]. However, another study could not confirm the nonredundant role of dectin-1 in *C. albicans* infection [104]. The role of dectin-1 in mucosal candidiasis has not been established yet, but several facts suggest that dectin-1 plays a crucial role in the mucosal immunity against *Candida*. First of all, dectin-1 is expressed in the intestinal tract, and the outgrowth of *Candida* in the digestive tract from dectin1 -/- mice was disproportionally high, leading to occlusion and contributing to the increased mortality [103,105,106]. Secondly, dectin-1 is paramount for IL-17 induction by *Candida* [94,107], and patients with an impaired IL-17 production caused by STAT3 mutations (hyper-IgE syndrome) and chronic mucocutaneous candidiasis have recurrent *Candida* infections [59-61].
No data are currently available on the role of dectin-1 in vaccination against *C. albicans*, although one would expect that engagement of dectin-1 will lead to a cytokine response inducing Th1 and Th17, which are both protective against *Candida*. Moreover, β-glucans are not only a cell wall component of all fungi but also occur abundantly in some algae, plants and mushrooms, and have been used for their immunomodulatory property for a long time [108,109]. It has been therefore hypothesized that β-glucans can exert patent adjuvant effects, presumably through interaction with dectin-1. However, the usage of β-glucans as adjuvants has to consider the chemical and biological properties of the various β-glucan preparations. The most commonly used β-glucan preparation is zymosan, a particle of the cell-wall of *Saccharomyces cerevisiae,* containing besides β-glucan also components that activate MR

and TLR2 signal pathways. The consequence of using this stimulus is parallel activation of different signal pathways. By using a soluble β-glucan that does not induce activation of the cell by itself, engagement of zymosan to the β-glucan receptor could be inhibited. This method indicates differences between soluble β-glucans that are biological inert and can even prevent binding to dectin-1 and phagocytosis, and particulated β-glucans that induce phagocytosis and activate signal pathways. Not only there are differences between soluble and particulated β-glucans, but the size of the particles also influences the immunological response [109]. Curdlan is a β-1,3-glucan derived from *Alcaligenes feacalis* that is used as a pure dectin-1 ligand. The larger particles obtained from curdlan induced higher production of pro-inflammatory cytokines in mBMDC compared to the smaller pure β-1,3-glucan particles obtained from *S. cerevisiae.* This difference in cytokine response largely depended on particle size, because the difference in cytokine production disappeared when curdlan was sonicated to the same particle size as the particles obtained from *S. cerevisiae* [91]. These differences in response caused by the nature of β-glucan should be taken in account when targeting dectin-1 for potential adjuvant activity in vaccination.

Dectin-2

Dectin-2 is a type II transmembrane receptor with a single CRD and a stalk region, but without an intracellular signaling motif [110]. Dectin-2 is expressed on macrophages and dendritic cells and is up-regulated when these cells are stimulated with particles containing high-mannose structures like *C. albicans* hyphae [111,112]. The specific ligand for dectin-2 is not known. Dectin-2 signaling occurs in collaboration with FcRγ chain and activates NF-κB and can induce IL-1Ra and TNF production [113]. No data on dectin-2 involvement in *Candida* reinfection or vaccination models are currently available.

Macrophage mannose receptor

The macrophage mannose receptor (MR) is a type I transmembrane receptor with an extracellular domain existing of a fibronectin type II domain, a cysteine-rich domain, eight CRDs and a short cytoplasmic tail. The CRD 4-8 function as binding site for PAMPs and can be activated by terminal mannose, fucose and N-acetyl glucosamine leading to Ca++ dependent signaling [114-116]. MR is also expressed intracellularly and can be shed from the cell membrane. Both macrophages and dendritic cells (DC) express MR [117-119]. Ligation of MR can induce phagocytosis of fungi, although this has been recently challenged in case of *C. albicans* [120]. Because MR has no known cytoplasmic signaling motif, there is not much known about the signal pathways activated by engagement of MR. Cross-linkage of MR with an activating anti-MR-antibody induced cytokine (IL-10 and IL-1Ra) production in DC, and up-regulation of co-stimulatory molecules leading to Th2 polarization in vitro [121]. MR engagement by mannans of *M. tuberculosis* also induced a Th2 immune response, by inhibiting production of Th1 cytokines [121,122]**.** From these data one could conclude that MR binding leads to Th2 polarization. However, mannans on pathogens are complex molecules and their biological effects can differ depending on the structure [123,124]**.** On the outer layer of

the cell-wall, *C. albicans* expresses N-linked phosphomannans and their binding to MR on human DC lead to internalization and cytokine production [124,125]. Human mononuclear cells produced Th1 cytokines after stimulation with *Candida* in a MR-dependent manner [79]. Also in murine models engagement of MR by *C. albicans* induced the proinflammatory cytokines IL-1β, IL-6, and GM-CSF [126], and MR deficiency led to a reduction of TNFα and MCP-1 release in response to *C. albicans* uptake [127]. Intra-peritoneal infection of MR deficient mice lead to a higher fungal burden compared to their WT littermates, although it did not influence mortality [128]. The general conclusion that can be drawn from these studies is that engagement of MR by *C. albicans* mannans induces proinflammatory cytokine responses that result in a predominant cellular Th1 response. There are no data published on the role of MR in reinfection models. If an adjuvant MR-ligand would be used, the structure of the candidate mannans to be employed may be crucial, as shown by the differences in cytokine profiles induced by the mannans of different microorganisms. In addition, the chemical treatment of mannans influences biological responses [129].

DC-SIGN and Mincle

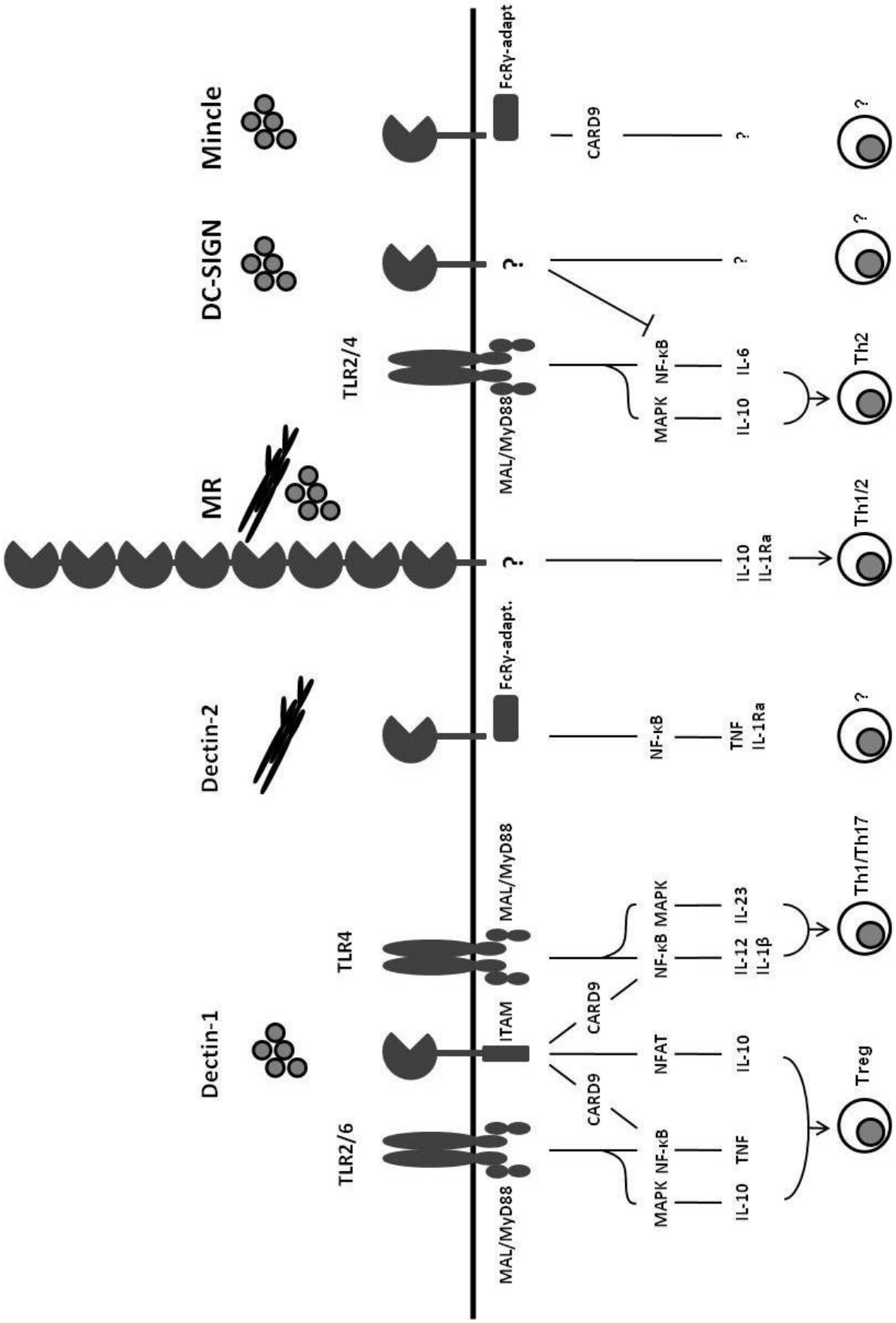
DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) is a type II transmembrane receptor with a single CRD, a stalk region and a cytoplasmic tail with signal motifs. DC-SIGN can form multimers and activate internalization signal pathways. DC-SIGN is expressed on dendritic cells and endothelium. Ligation with high-mannose structures leads to Ca++ dependent activation and tetramerisation of the receptor [130]. The role of DC-SIGN in phagocytosis is disputable, whereas induction of endocytosis and uptake of pathogens is well established [131]. DC-SIGN activates intracellular signal pathways involving Raf-kinase, ERK1 and ERK2, leading to cytokine production and modulation of TLR signal pathways. Activation of these signal pathways leads to an enhanced IL-10 production, thereby favoring a Th2 cellular immune response [132,133]. The uptake of *C. albicans* by human dendritic cells depends on the binding of N-linked mannan to DC-SIGN and induces IL-6 production [124,134]. Stimulation of DC-SIGN signal pathways for immunomodulation in vaccination is therefore likely to elicit mainly Th2 responses, limiting the usefulness of DC-SIGN as target for adjuvant effects.
Mincle (macrophage-inducible C-type lectin) is a type II transmembrane protein with a single CRD, a short stalk region and an intracellular region that signals by association with FcRγ adaptor and activates the Syk-CARD9 pathway [135,136]. Both human and mouse Mincle have been recently shown to bind *C. albicans* and contribute to cytokine stimulation [137] and Mincle deficient mice were more susceptible to systemic candidiasis [138]. Nothing is known about the role of Mincle in reinfection or vaccination models.

Cross-talk between dectin-1 signals and TLR-induced inflammatory pathways

Activation of inflammation leads to phagocytosis and killing of microorganisms, production of cytokines, migration, maturation of APCs and activation of specific of T-cells subsets. As microorganisms can activate more pathways simultaneously by ligation of different PRRs,

interaction between these pathways will occur and could induce a certain degree of specificity in the innate immune response to pathogens. Besides the MyD88-dependent interaction between dectin-1 and TLR2 for the induction of proinflammatory cytokines in macrophages, it has also been shown that dectin-1 signal pathways synergize with other TLRs. Particulated pure β-glucan derived from *S. cerevisiae* induced synergistic effects on the production of TNF, MIP-1α and MIP-2 when murine macrophages were stimulated in combination with Pam3Cys (TLR2-ligand), LPS (TLR4), flagellin (TLR5), CL-097 (TLR7) and ODN1826 (TLR9). These effects were exerted in a SYK and MyD88-dependent way by enhanced translocation of NF-κB [139]. Stimulation of primary human cells with soluble curdlan in combination with Pam3Cys (TLR2) or LPS (TLR4) induced synergistic IL-10 and TNFα production in a dectin-1-dependent manner [140]. In human monocyte-derived DCs (mono-DC), simultaneous stimulation with β-glucan and Pam2Cys (TLR2) or R848 (TLR8) induced synergistic IL-23 production, resulting in enhanced induction of IL-17 by co-cultured CD4+T-cells [141]. In human mono-DC, β-glucan did not induce IL-12p75 in absence of R848 (TLR8) and the induction could be inhibited by TLR2 stimulation [141]. Similarly, stimulation of murine DC with β-glucan did not induce IL-12p75 in the absence of INFγ, indicating interaction between dectin-1 and INFγ signal pathways [97]. These insights into the collaboration between TLR- and CLR-induced inflammatory pathways are therefore crucial for development of effective adjuvants and vaccine design (Figure 3). Therefore, an efficient adjuvant for *Candida* vaccination should probably be able to stimulate several of these receptors.

Figure 3. C-type lectin receptor signal pathways activated by *C. albicans* and induction of cellular responses. Cell-wall components of *C. albicans* blastoconidia and hyphae can activate C-type lectin receptor signal pathways that induce cytokine production and regulation of T-cell proliferation. Engagement by dectin-1 and DC-SIGN can modulate TLR pathways and enhance or inhibit cytokine responses. Ligation of dectin-1 leads to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM)-like motif and activation of a protease cascade containing CARD9 leading to translocation of NF-κB or NFAT. The dectin-2 and Mincle signaling cascade is activated by association of the Fcγ receptor-adaptor molecule (FcγR-adapt).


Dectin-1
TLR2/6
MAL/MyD88
MAPK NF-κB
IL-10 TNF
CARD9
ITAM
NFAT
IL-10
Treg
TLR4
MAL/MyD88
CARD9
NF-κB MAPK
IL-12 IL-23
IL-1β
Th1/Th17
Dectin-2
FcRγ-adapt.
NF-κB
TNF
IL-1Ra
?
MR
?
IL-10
IL-1Ra
Th1/2
TLR2/4
MAL/MyD88
MAPK NF-κB
IL-10 IL-6
Th2
DC-SIGN
?
?
?
Mincle
FcRγ-adapt
CARD9
?
?

Concluding remarks and future directions

Recent progress has provided important novel insights in the processes driving the adaptive immune responses. Central to these developments is the discovery of pattern recognition receptors like TLRs and CLRs that not only induce innate immune responses, but also modulate cellular and humoral adaptive immunity. As vaccination is one of the great achievements in medicine and probably the most powerful tool to protect human and animals against infectious disease, further vaccine development and optimization of current strategies can improve health status of large groups of people. Development of a vaccine against *Candida spp.* should induce both cellular and humoral immune responses. While the TLRs are strong inducers of inflammatory responses, it seems that the CLRs have the potential to modulate these responses by enhancement or inhibition of cytokine production. Understanding the natural host defense mechanisms against pathogens like *C. albicans* therefore helps to identify the proper targets for inducing a strong adjuvant effect, in order to stimulate an effective adaptive immune response and protection.

An exciting new development is the discovery that specific human gene mutations and polymorphisms are linked to signal pathways resulting in susceptibility for specific pathogens. STAT3 mutations identified in patients with hyper IgE syndrome have recently been linked to a defective IL-17 production and a diminished Th17 response, resulting in recurrent mucosal *Candida* infections. Similarly, we have recently described a family with defective dectin-1 expression due to an early stop-codon mutation leading to an increased susceptibility to mucosal *Candida* infection [142], and a similar phenotype has been described in a family with CARD9 deficiency [143]. Linkage of genetics to functional immunological data proves therefore to be a very powerful research tool.

A very important task of future studies will be to identify the adjuvant activity of specific PRR ligands in context of *Candida* vaccination. Combinations of various candidate vaccines with the known TLR/CLR adjuvants should be tested. In addition, new adjuvants containing combinations of TLR and CLR specificities should be designed and tested. Through these strategies, we should be able to develop new and potent vaccine candidates against *Candida* infections in the coming years.

Reference List

1 Nucci,M. and Marr,K.A. (2005) Emerging fungal diseases. *Clin. Infect. Dis.* 41, 521-526

2 Edmond,M.B. *et al.* (1999) Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Infect. Dis.* 29, 239-244

3 Wisplinghoff,H. *et al.* (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* 39, 309-317

4 Pfaller,M.A. and Diekema,D.J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* 20, 133-163

5 McNeil,M.M. *et al.* (2001) Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin. Infect. Dis.* 33, 641-647

6 Enoch,D.A. *et al.* (2006) Invasive fungal infections: a review of epidemiology and management options. *J. Med. Microbiol.* 55, 809-818

7 Cassone,A. (2008) Fungal vaccines: real progress from real challenges. *Lancet Infect. Dis.* 8, 114-124

8 Ada,G. (2001) Vaccines and vaccination. *N. Engl. J. Med.* 345, 1042-1053

9 Del,G.G. (2003) Vaccination strategies. An overview. *Vaccine* 21 Suppl 2, S83-S88

10 Berzofsky,J.A. *et al.* (2001) Strategies for designing and optimizing new generation vaccines. *Nat. Rev. Immunol.* 1, 209-219

11 Pashine,A. *et al.* (2005) Targeting the innate immune response with improved vaccine adjuvants. *Nat. Med.* 11, S63-S68

12 Hoebe,K. *et al.* (2004) The interface between innate and adaptive immunity. *Nat. Immunol.* 5, 971-974

13 Akira,S. and Takeda,K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* 4, 499-511

14 Akira,S. *et al.* (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2, 675-680

15 Kapsenberg,M.L. (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* 3, 984-993

16 Napolitani,G. *et al.* (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6, 769-776

17 Kubin,M. *et al.* (1994) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med.* 180, 211-222

18 Guy,B. (2007) The perfect mix: recent progress in adjuvant research. *Nat. Rev. Microbiol.* 5, 505-517

19 Lindblad,E.B. (2004) Aluminium compounds for use in vaccines. *Immunol. Cell Biol.* 82, 497-505

20 Eisenbarth,S.C. *et al.* (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453, 1122-1126

21 Kool,M. *et al.* (2008) Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* 181, 3755-3759

22 Mosca,F. *et al.* (2008) Molecular and cellular signatures of human vaccine adjuvants. *Proc. Natl. Acad. Sci. U. S. A* 105, 10501-10506

23 Tritto,E. *et al.* (2009) Mechanism of action of licensed vaccine adjuvants. *Vaccine* 27, 3331-3334

24 McCluskie,M.J. and Krieg,A.M. (2006) Enhancement of infectious disease vaccines through TLR9-dependent recognition of CpG DNA. *Curr. Top. Microbiol. Immunol.* 311, 155-178

25 Pankhurst,C. and Peakman,M. (1989) Reduced CD4 + T cells and severe oral candidiasis in absence of HIV infection. *Lancet* 1, 672

26 Klein,R.S. *et al.* (1984) Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 311, 354-358

27 Vogt,F.C. (1979) The incidence of oral candidiasis with use of inhaled corticosteroids. *Ann. Allergy* 43, 205-210

28 Farah,C.S. *et al.* (2002) Primary role for CD4(+) T lymphocytes in recovery from oropharyngeal candidiasis. *Infect. Immun.* 70, 724-731

29 Farah,C.S. *et al.* (2001) T cells augment monocyte and neutrophil function in host resistance against oropharyngeal candidiasis. *Infect. Immun.* 69, 6110-6118

30 Ibrahim,A.S. *et al.* (2005) Vaccination with recombinant N-terminal domain of Als1p improves survival during murine disseminated candidiasis by enhancing cell-mediated, not humoral, immunity. *Infect. Immun.* 73, 999-1005

31 Matthews,R.C. *et al.* (1987) Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to Candida albicans. *J. Clin. Microbiol.* 25, 230-237

32 Lopez-Ribot,J.L. *et al.* (2004) Antibody response to Candida albicans cell wall antigens. *FEMS Immunol. Med. Microbiol.* 41, 187-196

33 Zhang,M.X. *et al.* (1997) Mannan-specific immunoglobulin G antibodies in normal human serum mediate classical pathway initiation of C3 binding to Candida albicans. *Infect. Immun.* 65, 3822-3827

34 Zhang,M.X. *et al.* (1998) Contrasting roles of mannan-specific monoclonal immunoglobulin M antibodies in the activation of classical and alternative pathways by Candida albicans. *Infect. Immun.* 66, 6027-6029

35 Wellington,M. *et al.* (2007) Monocyte responses to Candida albicans are enhanced by antibody in cooperation with antibody-independent pathogen recognition. *FEMS Immunol. Med. Microbiol.* 51, 70-83

36 Wagner,R.D. *et al.* (1996) B cell knockout mice are resistant to mucosal and systemic candidiasis of endogenous origin but susceptible to experimental systemic candidiasis. *J. Infect. Dis.* 174, 589-597

37 Park,M.A. *et al.* (2008) Common variable immunodeficiency: a new look at an old disease. *Lancet* 372, 489-502

38 Cunningham-Rundles,C. and Bodian,C. (1999) Common variable immunodeficiency: clinical and immunological features of 248 patients. *Clin. Immunol.* 92, 34-48

39 Ashman,R.B. *et al.* (2004) Innate versus adaptive immunity in Candida albicans infection. *Immunol. Cell Biol.* 82, 196-204

40 Casadevall,A. *et al.* (2002) Induced humoral immunity and vaccination against major human fungal pathogens. *Curr. Opin. Microbiol.* 5, 386-391

41 Farah,C.S. and Ashman,R.B. (2005) Active and passive immunization against oral Candida albicans infection in a murine model. *Oral Microbiol. Immunol.* 20, 376-381

42 Spellberg,B.J. *et al.* (2006) Efficacy of the anti-Candida rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* 194, 256-260

43 Han,Y. *et al.* (1999) Candida albicans mannan extract-protein conjugates induce a protective immune response against experimental candidiasis. *J. Infect. Dis.* 179, 1477-1484

44 Vilanova,M. *et al.* (2004) Protection against systemic candidiasis in mice immunized with secreted aspartic proteinase 2. *Immunology* 111, 334-342

45 Yang,Q. *et al.* (2005) Prophylactic vaccination with phage-displayed epitope of C. albicans elicits protective immune responses against systemic candidiasis in C57BL/6 mice. *Vaccine* 23, 4088-4096

46 Mencacci,A. *et al.* (1996) CD4+ T-helper-cell responses in mice with low-level Candida albicans infection. *Infect. Immun.* 64, 4907-4914

47 Cenci,E. *et al.* (1998) IFN-gamma is required for IL-12 responsiveness in mice with Candida albicans infection. *J. Immunol.* 161, 3543-3550

48 Villamon,E. *et al.* (2004) Toll-like receptor 2 is dispensable for acquired host immune resistance to Candida albicans in a murine model of disseminated candidiasis. *Microbes. Infect.* 6, 542-548

49 Fidel,P.L., Jr. *et al.* (1997) Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with Candida antigen. *J. Infect. Dis.* 176, 728-739

50 la,S.A. *et al.* (1996) Mannoproteins from Candida albicans elicit a Th-type-1 cytokine profile in human Candida specific long-term T cell cultures. *J. Biol. Regul. Homeost. Agents* 10, 8-12

51 Vonk,A.G. *et al.* (2006) Host defence against disseminated Candida albicans infection and implications for antifungal immunotherapy. *Expert. Opin. Biol. Ther.* 6, 891-903

52 Savolainen,J. *et al.* (1996) Enhanced IgE response to Candida albicans in postoperative invasive candidiasis. *Clin. Exp. Allergy* 26, 452-460

53 Zhou,M. *et al.* (2008) Memory Th-17 cells specific for C. albicans are persistent in human peripheral blood. *Immunol. Lett.* 118, 72-81

54 costa-Rodriguez,E.V. *et al.* (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8, 639-646

55 Huang,W. *et al.* (2004) Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J. Infect. Dis.* 190, 624-631

56 Zelante,T. *et al.* (2007) IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* 37, 2695-2706

57 Bozza,S. *et al.* (2008) Lack of Toll IL-1R8 exacerbates Th17 cell responses in fungal infection. *J. Immunol.* 180, 4022-4031

58 De,L.A. *et al.* (2007) Functional yet balanced reactivity to Candida albicans requires TRIF, MyD88, and IDO-dependent inhibition of Rorc. *J. Immunol.* 179, 5999-6008

59 Milner,J.D. *et al.* (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452, 773-776

60 Eyerich,K. *et al.* (2008) Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. *J. Invest Dermatol.* 128, 2640-2645

61 Ma,C.S. *et al.* (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J. Exp. Med.* 205, 1551-1557

62 Netea,M.G. *et al.* (2004) Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J. Immunol.* 172, 3712-3718

63 Sutmuller,R.P. *et al.* (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest* 116, 485-494

64 Vignali,D.A. *et al.* (2008) How regulatory T cells work. *Nat. Rev. Immunol.* 8, 523-532

65 Netea,M.G. *et al.* (2008) An integrated model of the recognition of Candida albicans by the innate immune system. *Nat. Rev. Microbiol.* 6, 67-78

66 Pasare,C. and Medzhitov,R. (2005) Control of B-cell responses by Toll-like receptors. *Nature* 438, 364-368

67 Iwasaki,A. and Medzhitov,R. (2004) Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5, 987-995

68 Nemazee,D. *et al.* (2006) Immunology: Toll-like receptors and antibody responses. *Nature* 441, E4

69 Gavin,A.L. *et al.* (2006) Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 314, 1936-1938

70 Jouault,T. *et al.* (2003) Candida albicans phospholipomannan is sensed through toll-like receptors. *J. Infect. Dis.* 188, 165-172

71 Netea,M.G. *et al.* (2002) The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J. Infect. Dis.* 185, 1483-1489

72 Blasi,E. *et al.* (2005) Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with Candida albicans. *FEMS Immunol. Med. Microbiol.* 44, 69-79

73 Villamon,E. *et al.* (2004) Toll-like receptor-2 is essential in murine defenses against Candida albicans infections. *Microbes. Infect.* 6, 1-7

74 Van der Graaf,C.A. *et al.* (2005) Differential cytokine production and Toll-like receptor signaling pathways by Candida albicans blastoconidia and hyphae. *Infect. Immun.* 73, 7458-7464

75 Bellocchio,S. *et al.* (2004) The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J. Immunol.* 172, 3059-3069

76 d'Ostiani,C.F. *et al.* (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. *J. Exp. Med.* 191, 1661-1674

77 Takeuchi,O. *et al.* (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13, 933-940

78 Netea,M.G. *et al.* (2008) Role of TLR1 and TLR6 in the host defense against disseminated candidiasis. *FEMS Immunol. Med. Microbiol.* 52, 118-123

79 Netea,M.G. *et al.* (2006) Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest* 116, 1642-1650

80 Van der Graaf,C.A. *et al.* (2006) Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for Candida bloodstream infection. *Eur. Cytokine Netw.* 17, 29-34

81 Gil,M.L. and Gozalbo,D. (2006) TLR2, but not TLR4, triggers cytokine production by murine cells in response to Candida albicans yeasts and hyphae. *Microbes. Infect.* 8, 2299-2304

82 Netea,M.G. *et al.* (2006) Both TLR2 and TLR4 are involved in the recognition of Candida albicans. Reply to "TLR2, but not TLR4, triggers cytokine production by murine cells in response to Candida

albicans yeasts and hyphae" by Gil and Gozalbo, Microbes and Infection 8 (2006) 2823-2824. *Microbes. Infect.* 8, 2821-2822

83 van,d., V *et al.* (2008) Redundant role of TLR9 for anti-Candida host defense. *Immunobiology* 213, 613-620

84 Bozza,S. *et al.* (2002) Vaccination of mice against invasive aspergillosis with recombinant Aspergillus proteins and CpG oligodeoxynucleotides as adjuvants. *Microbes. Infect.* 4, 1281-1290

85 Zelensky,A.N. and Gready,J.E. (2005) The C-type lectin-like domain superfamily. *FEBS J.* 272, 6179-6217

86 Willment,J.A. and Brown,G.D. (2008) C-type lectin receptors in antifungal immunity. *Trends Microbiol.* 16, 27-32

87 Ariizumi,K. *et al.* (2000) Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* 275, 20157-20167

88 Willment,J.A. *et al.* (2001) Characterization of the human beta -glucan receptor and its alternatively spliced isoforms. *J. Biol. Chem.* 276, 43818-43823

89 Rogers,N.C. *et al.* (2005) Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity.* 22, 507-517

90 Underhill,D.M. *et al.* (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* 106, 2543-2550

91 Rosas,M. *et al.* (2008) The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis. *J. Immunol.* 181, 3549-3557

92 Gross,O. *et al.* (2006) Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442, 651-656

93 Hara,H. *et al.* (2007) The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. *Nat. Immunol.* 8, 619-629

94 LeibundGut-Landmann,S. *et al.* (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8, 630-638

95 Brown,G.D. *et al.* (2003) Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197, 1119-1124

96 Gantner,B.N. *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107-1117

97 Dillon,S. *et al.* (2006) Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Invest* 116, 916-928

98 Goodridge,H.S. *et al.* (2007) Dectin-1 stimulation by Candida albicans yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J. Immunol.* 178, 3107-3115

99 Herre,J. *et al.* (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104, 4038-4045

100 Hall,A. (1998) Rho GTPases and the actin cytoskeleton. *Science* 279, 509-514

101 Torosantucci,A. *et al.* (2009) Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. *PLoS. ONE.* 4, e5392

102 Gantner,B.N. *et al.* (2005) Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *EMBO J.* 24, 1277-1286

103 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

104 Saijo,S. *et al.* (2007) Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat. Immunol.* 8, 39-46

105 Reid,D.M. *et al.* (2004) Expression of the beta-glucan receptor, Dectin-1, on murine leukocytes in situ correlates with its function in pathogen recognition and reveals potential roles in leukocyte interactions. *J. Leukoc. Biol.* 76, 86-94

106 Taylor,P.R. *et al.* (2002) The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.* 169, 3876-3882

107 Osorio,F. *et al.* (2008) DC activated via dectin-1 convert Treg into IL-17 producers. *Eur. J. Immunol.* 38, 3274-3281

108 Borchers,A.T. *et al.* (2004) Mushrooms, tumors, and immunity: an update. *Exp. Biol. Med. (Maywood.)* 229, 393-406

109 Brown,G.D. and Gordon,S. (2003) Fungal beta-glucans and mammalian immunity. *Immunity.* 19, 311-315

110 Ariizumi,K. *et al.* (2000) Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem.* 275, 11957-11963

111 McGreal,E.P. *et al.* (2006) The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* 16, 422-430

112 Taylor,P.R. *et al.* (2005) Dectin-2 is predominantly myeloid restricted and exhibits unique activation-dependent expression on maturing inflammatory monocytes elicited in vivo. *Eur. J. Immunol.* 35, 2163-2174

113 Sato,K. *et al.* (2006) Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J. Biol. Chem.* 281, 38854-38866

114 Taylor,M.E. (2001) Structure and function of the macrophage mannose receptor. *Results Probl. Cell Differ.* 33, 105-121

115 Taylor,M.E. *et al.* (1990) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. *J. Biol. Chem.* 265, 12156-12162

116 Taylor,M.E. *et al.* (1992) Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. *J. Biol. Chem.* 267, 1719-1726

117 Taylor,P.R. *et al.* (2005) Macrophage receptors and immune recognition. *Annu. Rev. Immunol.* 23, 901-944

118 Taylor,P.R. *et al.* (2005) The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol.* 26, 104-110

119 McKenzie,E.J. *et al.* (2007) Mannose receptor expression and function define a new population of murine dendritic cells. *J. Immunol.* 178, 4975-4983

120 Le,C., V *et al.* (2005) The human macrophage mannose receptor is not a professional phagocytic receptor. *J. Leukoc. Biol.* 77, 934-943

121 Chieppa,M. *et al.* (2003) Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J. Immunol.* 171, 4552-4560

122 Nigou,J. *et al.* (2001) Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J. Immunol.* 166, 7477-7485

123 Lam,J.S. *et al.* (2007) Effect of differential N-linked and O-linked mannosylation on recognition of fungal antigens by dendritic cells. *PLoS. ONE.* 2, e1009

124 Cambi,A. *et al.* (2008) Dendritic cell interaction with Candida albicans critically depends on N-linked mannan. *J. Biol. Chem.* 283, 20590-20599

125 Newman,S.L. and Holly,A. (2001) Candida albicans is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect. Immun.* 69, 6813-6822

126 Yamamoto,Y. *et al.* (1997) Involvement of mannose receptor in cytokine interleukin-1beta (IL-1beta), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1beta (MIP-1beta), MIP-2, and KC responses, caused by attachment of Candida albicans to macrophages. *Infect. Immun.* 65, 1077-1082

127 Heinsbroek,S.E. *et al.* (2008) Stage-specific sampling by pattern recognition receptors during Candida albicans phagocytosis. *PLoS. Pathog.* 4, e1000218

128 Lee,S.J. *et al.* (2003) Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect. Immun.* 71, 437-445

129 Stambas,J. *et al.* (2002) Oxidised mannan-listeriolysin O conjugates induce Th1/Th2 cytokine responses after intranasal immunisation. *Vaccine* 20, 1877-1886

130 Koppel,E.A. *et al.* (2005) Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell Microbiol.* 7, 157-165

131 Taylor,P.R. *et al.* (2004) The role of SIGNR1 and the beta-glucan receptor (dectin-1) in the nonopsonic recognition of yeast by specific macrophages. *J. Immunol.* 172, 1157-1162

132 Caparros,E. *et al.* (2006) DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. *Blood* 107, 3950-3958

133 Gringhuis,S.I. *et al.* (2007) C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity.* 26, 605-616

134 Cambi,A. *et al.* (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *Eur. J. Immunol.* 33, 532-538

135 Balch,S.G. *et al.* (2002) Organization of the mouse macrophage C-type lectin (Mcl) gene and identification of a subgroup of related lectin molecules. *Eur. J. Immunogenet.* 29, 61-64

136 Yamasaki,S. *et al.* (2008) Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* 9, 1179-1188

137 Bugarcic,A. *et al.* (2008) Human and mouse macrophage-inducible C-type lectin (Mincle) bind Candida albicans. *Glycobiology* 18, 679-685

138 Wells,C.A. *et al.* (2008) The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. *J. Immunol.* 180, 7404-7413

139 Dennehy,K.M. *et al.* (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* 38, 500-506

140 Ferwerda,G. *et al.* (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol.* 10, 2058-2066

141 Gerosa,F. *et al.* (2008) Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J. Exp. Med.* 205, 1447-1461

142 Ferwerda,B. *et al.* (2009) Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* 361, 1760-1767

143 Glocker,E.O. *et al.* (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N. Engl. J. Med.* 361, 1727-1735

Part II

Cross-talk between TLRs and NLRs for pathogen recognition

Chapter 7

Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release

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Abstract

The recognition of peptidoglycan by cells of the innate immune system has been controversial; both TLR2 and nucleotide-binding oligomerization domain-2 (Nod2) have been implicated in this process. In the present study we demonstrate that although Nod2 is required for recognition of peptidoglycan, this leads to strong synergistic effects on TLR2-mediated production of both pro- and anti-inflammatory cytokines. Defective IL-10 production in patients with Crohn's disease bearing loss of function mutations of Nod2 may lead to overwhelming inflammation due to a subsequent Th1 bias. In addition to the potentiation of TLR2 effects, Nod2 is a modulator of signals transmitted through TLR4 and TLR3, but not through TLR5, TLR9, or TLR7. Thus, interaction between Nod2 and specific TLR pathways may represent an important modulatory mechanism of innate immune responses.

Introduction

Several years ago, a susceptibility locus for Crohn's disease on chromosome 16 was identified and named the IBD1 locus [1]; later, the candidate nucleotide-binding oligomerization domain-2 (Nod2) gene was identified within the IBD1 locus [2-4]. Because Nod2 is a member of the NOD-leucine-rich repeat protein family (also named the CATERPILLER family), which is known to be involved in recognition of microbial structures, and is expressed intracellularly in APCs [5], it has been hypothesized that Nod2 may be involved in pattern recognition of pathogens.

Initially, Nod2 was suggested to be an intracellular pattern recognition receptor for LPS [6], similar to the Nod1 [7], but later studies have demonstrated that Nod2 is the intracellular receptor for the muramyl dipeptide (MDP) component of peptidoglycan (PGN) [8,9]. However, other studies have shown that PGN stimulates cytokine production through TLR2, and it has been unclear whether Nod2 and TLR2 pathways are independent or interact with each other. The latter possibility has been suggested in patients with Crohn's disease homozygous for the 3020insC mutation of the Nod2 gene, who were found to have a defective release of cytokines not only after stimulation with MDP and PGN, but also after other TLR2 stimuli such as tripalmitoyl-S-glycerylcysteine (Pam3Cys) [10]. Moreover, the interaction between the Nod2 and TLR2 pathways is sustained by recent data from mice deficient in Nod2, which show down-regulation of TLR2-mediated Th1 responses by MDP [11].

In the present study we investigated the possible interaction between Nod2 and TLR pathways in human cells by studying the modulation of TLR-induced cytokine production by Nod2 signals activated by MDP. We assessed 1) the differential role of Nod2 and TLR2 for the induction of cytokines by *Staphylococcus aureus* PGN; 2) the synergistic effects of MDP on the cytokine production stimulated by the TLR2 ligands Pam3Cys and macrophage-activating lipopeptide 2 from Mycoplasma fermentation (MALP2); and 3) the functional consequences for the Nod2/TLR2 synergism of the Nod2 3020insC frameshift mutation in patients with Crohn's disease. In addition, we have investigated the possible interaction of Nod2 with other TLRs, namely the synergism of MDP/Nod2 with LPS (TLR4 signals), flagellin (TLR5), polyinosinic-polycytidylic (poly(I:C); TLR3), unmethylated CpG sequences of bacterial DNA (TLR9), and loxoribin (TLR7).

Results

Requirement for TLR2 and Nod2 for stimulation of cytokines by PGN

To investigate the involvement of TLR2 and Nod2 in the recognition of PGN, we stimulated cells isolated from mice deficient in TLR2 or from patients homozygous for the 3020insC mutation. TLR2–/– macrophages released significantly less TNF and IL-10 after stimulation with commercial PGN than control TLR2+/+ mice, whereas the production of TNF and IL-10 by TLR4-defective ScCr mice was intact (Fig. 1A). In contrast, stimulation with purified PGN, although less than that of commercial PGN, was independent of both TLR2 and TLR4 (Fig. 1B). In contrast, MNC isolated from patients homozygous for the 3020insC Nod2 mutation showed deficient production of TNF and IL-10 after stimulation with purified PGN (Fig. 2).

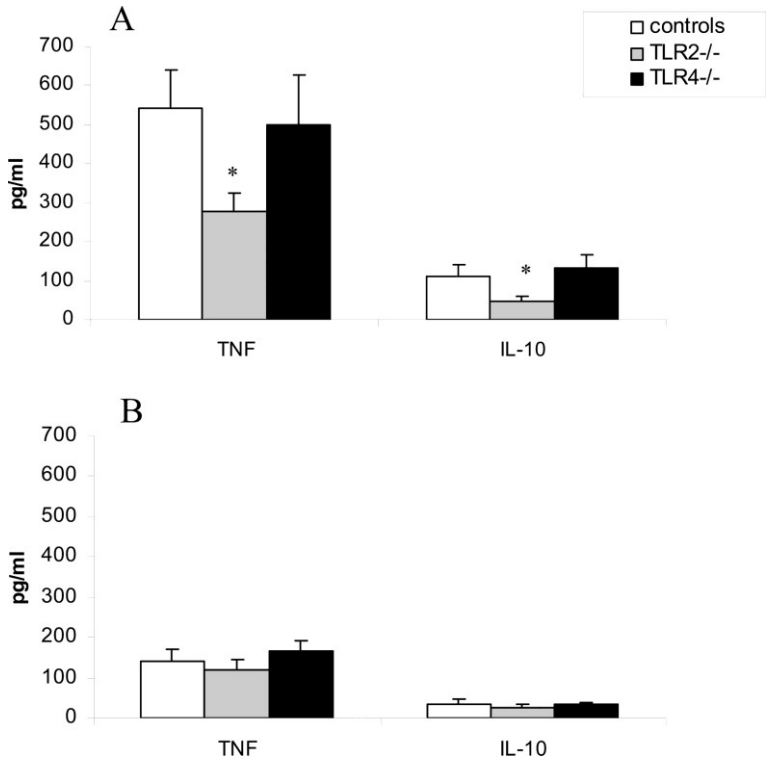

Figure 1. PGN stimulation of cytokines: requirement for TLR2. Peritoneal macrophages isolated from either wild-type mice or mice deficient in TLR2 or TLR4 were stimulated with 1 µg/ml commercial PGN (A) or purified PGN (B) for 24 h at 37°C. TNF and IL-10 were measured by specific RIA and ELISA, respectively. Data are presented as the mean ± SD (n = 10/group) and were compared by the Mann-Whitney U test (*, $p < 0.05$).

The data showing the requirement for TLR2 only for the recognition of commercial PGN are further strengthened by the observations of CHO cells transfected with human TLR2. Stimulation with commercial PGN led to signal transduction and CD25 expression in TLR2-transfected cells, as did Pam3Cys, but not in cells transfected with human TLR4. In contrast, the purified PGN did not induce CD25 expression in either TLR2- or TLR4-transfected cells (not shown).

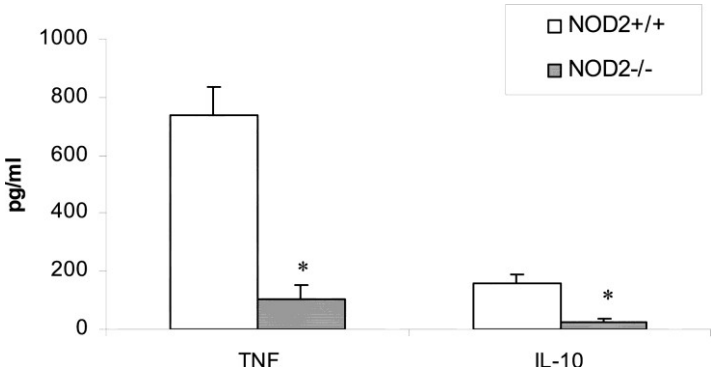

Figure 2. PGN stimulation of cytokines: requirement for Nod2. MNC isolated from four patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs) and five patients with the wild-type Nod2 allele were stimulated with 1 µg/ml purified PGN for 24 h at 37°C. TNF and IL-10 were measured by specific RIA and ELISA, respectively. Data are presented as the mean ± SD and were compared by the Mann-Whitney U test (*, $p < 0.05$).

Nod2 synergizes with TLR2 for production of cytokines

The data showing the requirement for both Nod2 and TLR2 for the production of cytokines by commercial PGN suggest an interaction between these two pathways. In line with this hypothesis, the specific Nod2 ligand MDP was found to have a synergistic effect on the induction of TNF, IL-1β, and IL-10 upon co-stimulation with the specific TLR2 agonists Pam3Cys (Fig. 3A) and MALP2 (Fig. 3B). In contrast, no IL-12p70 production was measured with any of the stimuli tested in both ELISAs used.

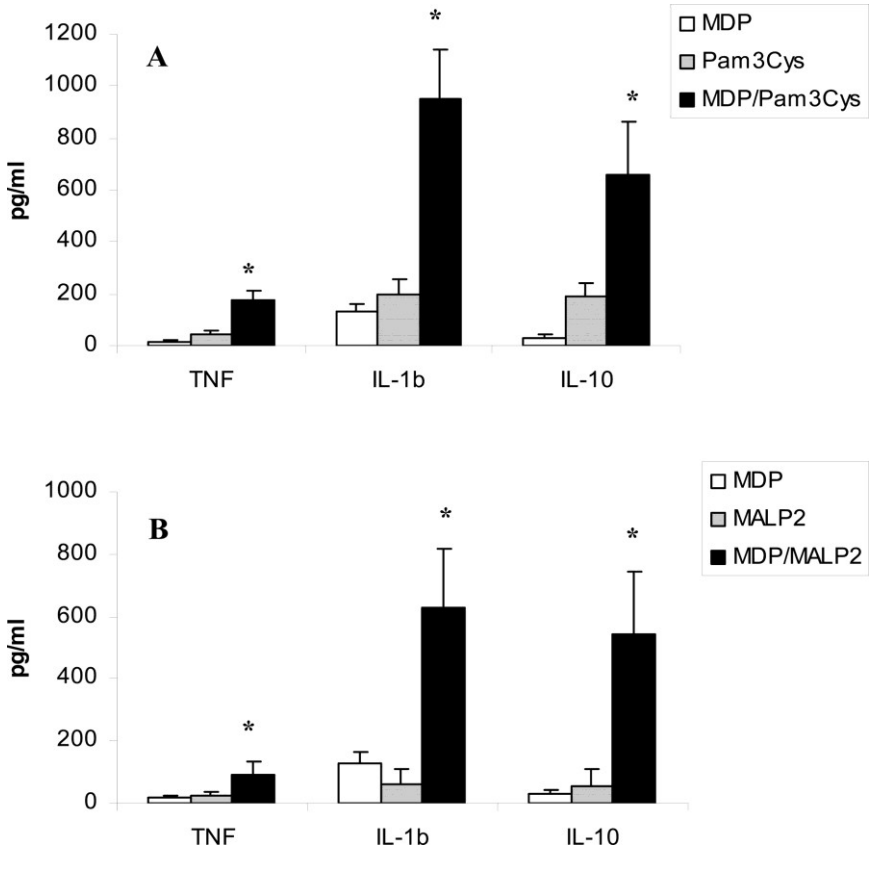

Figure 3. MDP synergizes with TLR2 agonists for the production of cytokines. MNC isolated from five healthy volunteers were stimulated with 10 µg/ml MDP, 1 µg/ml Pam3Cys or a combination of both (A). A similar combination of stimuli, but using MALP2 instead of Pam3Cys, is presented in B. After stimulation for 24 h at 37°C, TNF, IL-1, and IL-10 were measured by specific RIAs or ELISA, respectively. Data are presented as the mean ± SD, and were compared by the Mann-Whitney U test (*, $p < 0.05$).

To investigate whether MDP/Pam3Cys synergism is dependent on Nod2 and TLR2, we repeated the same experiment in patients bearing Nod2 mutations or mice deficient for TLR2. The MDP/Pam3Cys synergism was abrogated in patients homozygous for the 3020 insC mutation (Fig. 4). In addition, no synergism between MDP and Pam3Cys was observed in TLR2–/– mice (Fig. 5). These data demonstrate that both Nod2 receptors and TLR2 are required for the synergistic effect of MDP and Pam3Cys.

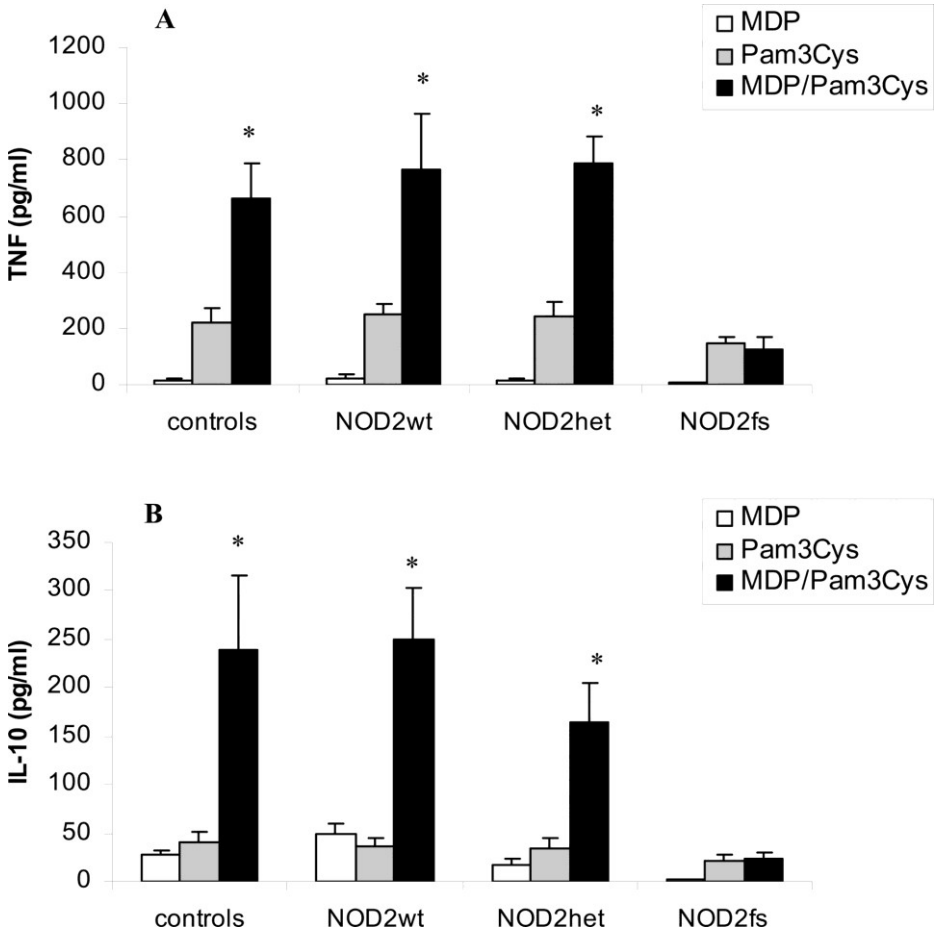

Figure 4. MDP/Pam3Cys synergistic stimulation of cytokine production depends on Nod2. MNC isolated from four patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs), five patients heterozygous for Nod2 mutations (Nod2het), five patients with the wild-type Nod2 allele (Nod2wt),and five healthy volunteers with wild-type Nod2 (controls) were stimulated with 10 µg/ml MDP, 1 µg/ml Pam3Cys or a combination of both (A and B). TNF and IL-10 were measured after 24-h stimulation at 37°C by specific RIA and ELISA, respectively. Data are presented as the mean ± SD, and were compared by the Wilcoxon paired test (*, $p < 0.05$).

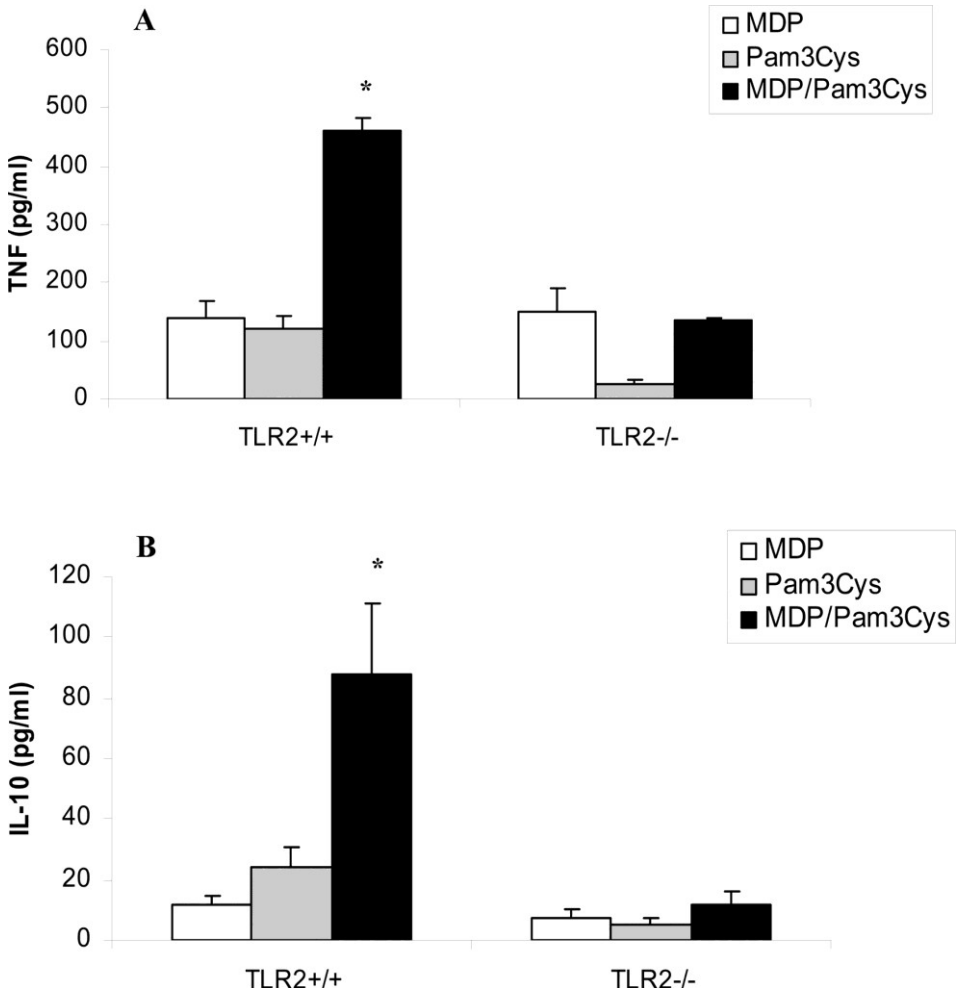

Figure 5. MDP/Pam3Cys synergistic stimulation of cytokine production depends on TLR2. Peritoneal macrophages isolated from either wild-type mice (TLR2+/+) or mice deficient in TLR2 (TLR2–/–) were stimulated with 10 μg/ml MDP, 1 μg/ml Pam3Cys or a combination of both. TNF and IL-10 were measured after 24-h stimulation at 37°C by specific RIA and ELISA, respectively. Data are presented as the mean ± SD and were compared by the Wilcoxon paired test (*, $p < 0.05$).

Nod2 modulates signaling via TLR4 and TLR3 pathways, but not TLR5-, TLR9-, and TLR7-mediated signals

Microorganisms display complex combinations of pathogen-associated molecular patterns (PAMPs) involved in simultaneous stimulations of more TLR pathways. We have investigated whether Nod2 modulates signals induced by various TLR agonists. Co-stimulation with the specific Nod2 ligand MDP potentiated the induction of TNF induced by LPS (TLR4 stimulation; Fig. 6A) and poly(I:C) (TLR3; Fig. 6C), whereas no effect was observed after stimulation with CpG (TLR9; Fig. 6B), flagellin (TLR5; Fig. 6D), or loxoribin (TLR7; not shown). The loss of synergistic activity in patients homozygous for the 3020insC Nod2 allele demonstrates that Nod2 is crucial for the potentiation of TLR4 and TLR3 signals by MDP (Fig. 6).

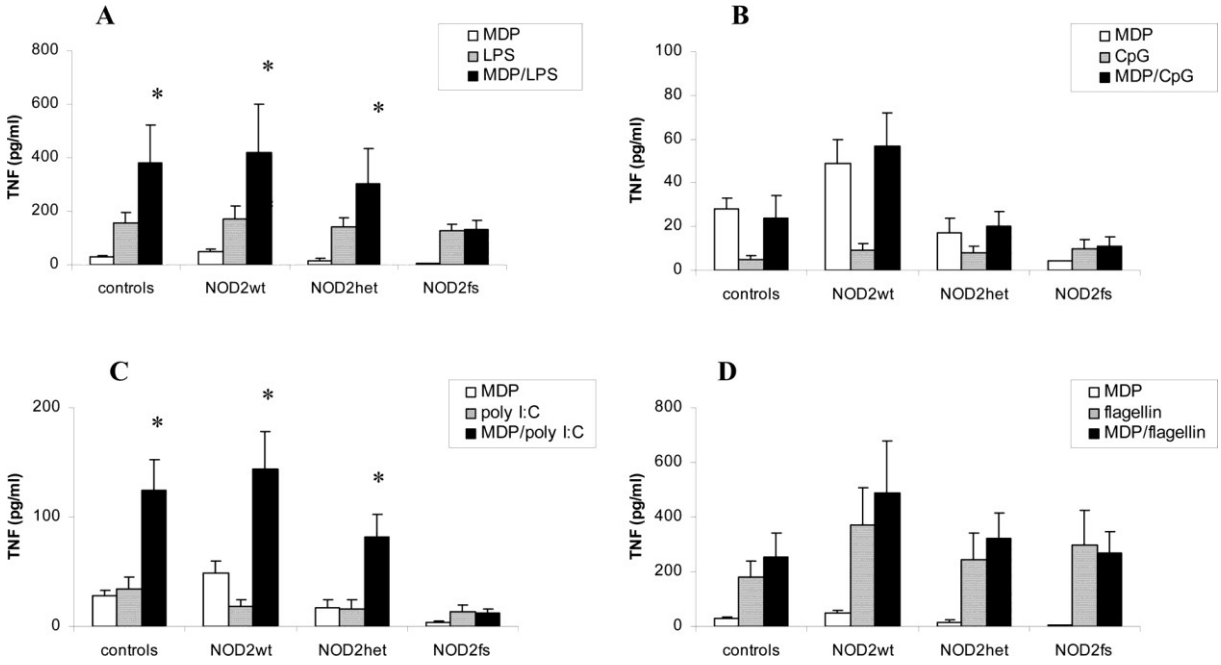

Figure 6. Nod2 signals potentiate TNF production induced by TLR4 and TLR3 ligands. MNC isolated from four patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs), five patients heterozygous for Nod2 mutations (Nod2het), five patients with the wild-type Nod2 allele (Nod2wt), and five healthy volunteers with wild-type Nod2 (controls) were stimulated with 10 µg/ml MDP, 1 ng/ml LPS (A), 5 µg/ml CpG motifs (B), 50 µg/ml poly(I:C) (C), or 10 ng/ml flagellin (D), either alone or in combination with MDP . TNF was measured after 24-h stimulation at 37°C by specific RIA. Data are presented as the mean ± SD and were compared by the Wilcoxon paired test (*, $p < 0.05$).

Discussion

In the present study we demonstrate that both TLR2 and Nod2 are required for the stimulation of cells by commercial PGN, whereas only Nod2 recognizes purified PGN. Nod2- and TLR2-mediated signals synergize at the level of proinflammatory and anti-inflammatory cytokine production, and this synergism is absent in patients with Crohn's disease who are homozygous for the Nod2 3020insC mutation. Patients with Crohn's disease bearing the 3020 insC mutation mainly display a defective release of the anti-inflammatory cytokine IL-10 [12]. In addition to its synergistic activity with TLR2, we have demonstrated that Nod2 modulates the intracellular signals induced by TLR4 and TLR3, but not by TLR5, TLR9, and TLR7 agonists.

There has been both confusion and controversy about whether recognition of PGN by the cells of the innate immune system occurs by surface TLR2 or by intracellular Nod2. Based on the studies performed in TLR2–/– mice, which are unresponsive to PGN and display enhanced susceptibility to Gram-positive microorganisms [13], it has initially been proposed that TLR2 is the cellular receptor for PGN. This assumption has later been challenged by the discovery of Nod2 as an intracellular receptor for the MDP component of PGN [14]. Nod2 mediates recognition of MDP and induces NF-κB activation after MDP challenge [15,16], and mice deficient in Nod2 fail to respond to a challenge with MDP [17]. In addition, mutations in Nod2 have been implicated in the pathogenesis of Crohn's disease [18,19], and patients homozygous for the 3020insC mutated allele have defective cellular responses to MDP and PGN [20]. The apparent discrepancy between the studies implicating either TLR2 or Nod2 for the recognition of PGN could be explained in two ways: firstly, by the contamination of commercial PGN by TLR2 ligands such as lipoteichoic acid, and secondly, by the cooperation between Nod2 and TLR2 pathways for the induction of cytokines. In the present study we demonstrate that although Nod2 recognizes purified PGN, both TLR2 and Nod2 are required for the efficient induction of proinflammatory cytokines by commercial PGN preparations, demonstrating that there is interaction between the TLR2 and Nod2 pathways.

A possible interaction between TLR2 and Nod2 pathways was also suggested by our observation that patients homozygous for the loss of function 3020insC Nod2 mutation are defective for the release of cytokines not only after PGN stimulation, but also after challenge with other TLR2 stimuli, such as Pam3Cys [21]. In the present study we demonstrate a synergism between Nod2- and TLR2-mediated signals, as shown by the synergistic effect of MDP on both Pam3Cys- and MALP2-induced TNF, IL-1β, and IL-10 production. The need for both receptors for this effect is demonstrated by the absence of synergism in patients bearing the 3020insC Nod2 mutation as well as in TLR2–/– mice. Our data regarding the interaction of the TLR2 and Nod2 pathways in human MNC are supported by earlier studies showing synergistic effects of MDP with PGN and lipoteichoic acid [22,23] and are reinforced by the findings of Watanabe et al. [24], who reported an interaction between Nod2 and TLR2 signals in murine macrophages. Interestingly, stimulation of the anti-inflammatory cytokine IL-10 by Pam3Cys, a specific TLR2 ligand, also seems to be decreased in patients with the Nod2 3020insC mutation. We observed this effect in a previous study [25], and this suggests

that Nod2 might exert some of its actions through directly mediating TLR2 signals. No such defects were observed when cells of the patients bearing the Nod2 mutation were stimulated with other TLR2 ligands.

Watanabe et al. [26] reported inhibitory effects of murine Nod2 signals on TLR2-induced Th1-type responses, as measured by IL-12 production. In our experiments using human Nod2-defective and control cells, IL-12 production was under the detection limit despite the use of stimulus concentrations as high as 10 μg/ml. We cannot confirm the inhibitory effect of Nod2 signals on Th1 responses found by Watanabe et al. [27] in murine cells, but a note of caution about their data should be mentioned, because the effects seen in their study were observed mainly at an MDP concentration of 100 μg/ml, which is hardly relevant for the in vivo situations. Another source of differences between our study and that of Watanabe et al. [28] is that they did not find differences in IL-10 release after PGN stimulation of murine Nod2–/– and Nod2+/+ cells [29]. This is an important observation, because it may underline important differences between the function of Nod2 in murine vs human cells and may explain the striking observation of the lack of intestinal inflammation in Nod2–/– mice [30] despite the crucial role of Nod2 in the development of Crohn's disease in humans. Alternatively, the observed differences between studies performed in human and murine cells may be caused by the presence of a defective Nod2 in patients, whereas a complete deletion of Nod2 was present in the knockout mice. Despite these differences, the synergistic interaction between Nod2 and TLR2 signals for the production of IL-10 in human MNC as well as the negative regulation of IL-12 production by Nod2, as shown by Watanabe et al. [31] with a shift toward protective Th2-type responses are probably important pathogenetic mechanisms in Crohn's disease.

The impact of Nod2 loss of function seems, therefore, to be related to the Th1/Th2 balance, rather than to the absolute defect in one of these cytokines. Thus, although production of the proinflammatory cytokines TNF and IL-1β is lower in Crohn's disease patients with the Nod2 3020 insC mutation, this effect seems to be over-run by the lack of protective Th2-type responses. This does not contradict the important role of TNF, because patients with Crohn's disease have been shown to have higher TNF production capacity and to be effectively treated by anti-TNF Abs [32]. An alternative explanation for the decreased proinflammatory cytokine production in patients with the mutation could be represented by defective antibacterial defense, leading to bacterial overgrowth and inflammation, as also suggested by others [33].

In addition to the interaction of Nod2-induced signals with TLR2, we demonstrate synergism of Nod2 with TLR4- and TLR3-mediated signals. Intestinal microorganisms are complex pathogens, with PAMPs requiring recognition by diverse TLRs, such as TLR2, TLR4, TLR5, and TLR9. From this perspective, interaction of the Nod2 pathway with other TLRs can be of considerable importance. In line with this idea, spontaneous enterocolitis associated with IL-12 overproduction in STAT3 knockout mice does not occur in the absence of TLR4 [34,35], and

TLR9 stimulation by CpG motifs increases the severity of dextran sodium sulfate-induced colitis [36].

Synergism between either PGN or MDP and bacterial LPS has been previously reported [37-39]. By showing the loss of MDP/LPS synergism in patients with the 3020insC Nod2 mutation, we demonstrate that this effect is mediated by the interaction between Nod2 and TLR4 pathways. In addition, we report synergism between Nod2 and TLR3; this interaction needs to be explored in models of intestinal inflammation.

The effects of Nod2 on TLR pathways seem to be selective, because we found no interaction with TLR5, TLR9, or TLR7. Despite the fact that TLR9 has been implicated in the pathogenesis of Crohn's disease based on experimental data [40,41] as well as the association of Crohn's disease with a TLR9 polymorphism [42], we could not find an interaction of Nod2 with the TLR9 pathway. Although the TLR5 agonist flagellin is an important PAMP of intestinal microorganisms, intestinal microvascular cells express TLR5, and bacterial flagellin has been suggested to be a dominant Ag in Crohn's disease [43,44], no interaction between Nod2 and TLR5 was detected. Likewise, there was no interaction between Nod2 and TLR7.

In conclusion, we report in this study that Nod2 enhances the signaling by TLR2, TLR4, and TLR3, whereas it does not influence the signals mediated by TLR5, TLR9, and TLR7. The interaction between Nod2 and TLRs is likely to be involved in the pathogenesis of Crohn's disease, because the lack of synergism in patients with loss of function mutations of Nod2 results in defective IL-10 production and a Th1 bias, effects that seem to over-ride the lower production of TNF and IL-1β. Studies are ongoing in our laboratory to decipher the precise intracellular mechanisms responsible for the Nod2/TLR synergism. Other studies have suggested the involvement of receptor-interacting protein 2 in the transduction of signals to both Nod2 and TLRs [45]. In addition, Wolfert et al. [46] proposed that the synergistic effect of MDP and LPS is exerted at the translational level; MDP is effective in transcribing DNA information in mRNA, but little translation of proteins takes place. Co-stimulation with specific (but not all) TLR ligands provides the signals able to translate the mRNA pool, leading to synergistic effects at the protein level. It remains to be demonstrated which of these mechanisms is responsible for the synergistic effects of these two pathways.

Materials and methods

Genotyping of Nod2 variants

Blood was collected from 74 patients with Crohn's disease and 10 healthy volunteers. PCR amplification of Nod2 gene fragments containing the polymorphic site 3020insC was performed in 50-µl reaction volumes containing 100–200 ng of genomic DNA, as previously described [47]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI PRISM 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems).

Four patients with Crohn's disease were found to be homozygous for the 3020insC mutation, and they were further investigated in the cytokine studies. As control groups, five patients with Crohn's disease heterozygous for the 3020 insC Nod2 mutation, five patients with Crohn's disease bearing the wild-type allele, and five healthy volunteers homozygous for the wild-type Nod2 allele were included.

Isolation of mononuclear cells and stimulation of cytokine production

After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10-ml EDTA tubes (Monoject; s-Hertogenbosch). Isolation of mononuclear cells (MNC) was performed as described previously [48] with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1/1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10 µg/ml gentamicin, 10 mM L-glutamine, and 10 mM pyruvate. The cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5×10^6 cells/ml.

MNC (5×10^5) in a 100-µl volume were added to round-bottom, 96-well plates (Greiner) and incubated with either 100 µl of culture medium (negative control) or the various stimuli (MDP (10 µg/ml; Sigma-Aldrich), commercial staphylococcal peptidoglycan (1 µg/ml; Sigma-Aldrich), purified peptidoglycan (gift from Dr. S. Girardin, Institute Pasteur, Paris, France), or synthetic Pam3Cys or MALP2 lipopeptides (1 µg/ml; EMC Microcollections)).

In separate experiments, stimulation of MNC with other TLR agonists was performed: TLR3 with poly(I:C) (50 µg/ml), TLR4 with purified Escherichia coli LPS (1 ng/ml), TLR5 with bacterial DNA (5 µg/ml), TLR5 with flagellin (10 ng/ml), TLR7 with loxoribin (5 µg/ml), and TLR9 with CpG (5 µg/ml). The synergism between Nod2 and TLR pathways was assessed using combinations of MDP with the various TLR stimuli. Submaximal concentrations of the TLR agonists were used in the experiments to allow better distinction of the synergistic effects with MDP among the various groups. The submaximal concentrations described above were determined in pilot experiments (not shown). All TLR agonists were checked for contamination with LPS in the Limulus amebocyte lysate assay and were found to be negative.

Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from either ScCr (TLR4-defective) or C57BL/10J (TLR4 control) mice and from either TLR2–/– or control TLR2+/+ mice (provided by Dr. S. Akira, Research Institute for Microbiol Diseases, Osaka University, Osaka, Japan) [49] were harvested by injection of 4 ml of sterile PBS containing 0.38% sodium citrate[50]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 µg/ml gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner) at 1 x 10^5 cells/well in a volume of 100 µl. The cells were stimulated with either purified *staphylococcal* peptidoglycan (1 µg/ml; commercial or purified) or a combination of MDP with Pam3Cys (both 1 µg/ml). After 24-h incubation at 37°C, the supernatants were collected and stored at –70°C until cytokine assays were performed.

Cytokine measurements

Human and murine TNFα and IL-1β concentrations were determined by specific RIAs as previously described [51,52]. IL-10 was measured by a commercial ELISA kit (Pelikine Compact; CLB) according to the instructions of the manufacturer. IL-12 concentrations were measured using two ELISA kits from R&D Systems and Pierce.

PGN signaling through human TLR2 in transfected cell lines

Chinese hamster ovary (CHO) fibroblasts stably transfected with human CD14 and TLR2 (3E10-TLR2) or TLR4 (3E10-TLR4), were a gift from Dr. R. Ingalls (Boston University, Boston, MA [53]). Cell lines express inducible membrane CD25 under control of a region from the human E-selectin (ELAM-1) promoter containing NF-B binding sites. Cells were maintained at 37°C and 5% CO2 in Ham's F-12 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 0.01% L-glutamine, 50 µg/ml gentamicin, 400 U/ml hygromycin, and 0.5 mg/ml G418 (for 3E10-TLR2) or 0.05 mg/ml puromycin (for 3E10-TLR4) as additional selection antibiotics. TLR2 and TLR4 expression was confirmed by flow cytometry (EPICS XL-MCL, Beckman Coulter) using PE-labeled anti-TLR2 (clone TL2.1) or anti-TLR4 (clone HTA125; Immunosource).

For stimulation experiments, 500 µl of cells in culture medium at a density of 1 x 10^5/ml were plated in 24-well culture plates. After an overnight incubation, cells were incubated with control medium, PGN (10 µg/ml), Pam3Cys (10 µg/ml), or LPS (1 µg/ml) for 20 h; thereafter, cells were harvested using trypsin/EDTA (Cambrex) and prepared for flow cytometry (Coulter FACScan). CD25 expression of the CHO cells was measured using FITC-labeled anti-CD25 (DakoCytomation).

Statistical analysis

The human experiments were performed in triplicate with blood obtained from patients and volunteers. The mouse experiments were performed twice in 10 mice/group, and the data are presented as the cumulative results of all experiments performed. The differences

between groups were analyzed by Mann-Whitney U test and, where appropriate, by Kruskal-Wallis ANOVA. The level of significance between groups was set at $p < 0.05$. The data are given as the mean ± SD.

Acknowledgements

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Reference List

1 Hugot,J.P. *et al.* (1996) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379, 821-823

2 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

3 Hampe,J. *et al.* (2002) Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 359, 1661-1665

4 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

5 Gutierrez,O. *et al.* (2002) Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J. Biol. Chem.* 277, 41701-41705

6 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

7 Inohara,N. *et al.* (2001) Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J. Biol. Chem.* 276, 2551-2554

8 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

9 Inohara,N. *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* 278, 5509-5512

10 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

11 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

12 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

13 Takeuchi,O. *et al.* (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J. Immunol.* 165, 5392-5396

14 Girardin,S.E. and Philpott,D.J. (2004) Mini-review: the role of peptidoglycan recognition in innate immunity. *Eur. J. Immunol.* 34, 1777-1782

15 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

16 Inohara,N. *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* 278, 5509-5512

17 Pauleau,A.L. and Murray,P.J. (2003) Role of nod2 in the response of macrophages to toll-like receptor agonists. *Mol. Cell Biol.* 23, 7531-7539

18 Hampe,J. *et al.* (2002) Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 359, 1661-1665

19 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

20 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

21 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

22 Wolfert,M.A. *et al.* (2002) The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan. *J. Biol. Chem.* 277, 39179-39186

23 Yang,S. *et al.* (2001) Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect. Immun.* 69, 2045-2053

24 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

25 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

26 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

27 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

28 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

29 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

30 Pauleau,A.L. and Murray,P.J. (2003) Role of nod2 in the response of macrophages to toll-like receptor agonists. *Mol. Cell Biol.* 23, 7531-7539

31 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

32 Targan,S.R. *et al.* (1997) A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N. Engl. J. Med.* 337, 1029-1035

33 Wehkamp,J. *et al.* (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53, 1658-1664

34 Kobayashi,M. *et al.* (2003) Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J. Clin. Invest* 111, 1297-1308

35 Kobayashi,M. *et al.* (2003) Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J. Clin. Invest* 111, 1297-1308

36 Obermeier,F. *et al.* (2002) CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur. J. Immunol.* 32, 2084-2092

37 Wolfert,M.A. *et al.* (2002) The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan. *J. Biol. Chem.* 277, 39179-39186

38 Yang,S. *et al.* (2001) Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect. Immun.* 69, 2045-2053

39 Traub,S. *et al.* (2004) Structural requirements of synthetic muropeptides to synergize with lipopolysaccharide in cytokine induction. *J. Biol. Chem.* 279, 8694-8700

40 Rachmilewitz,D. *et al.* (2004) Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 126, 520-528

41 Obermeier,F. *et al.* (2002) CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur. J. Immunol.* 32, 2084-2092

42 Torok,H.P. *et al.* (2004) Crohn's disease is associated with a toll-like receptor-9 polymorphism. *Gastroenterology* 127, 365-366

43 Maaser,C. *et al.* (2004) Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. *J. Immunol.* 172, 5056-5062

44 Lodes,M.J. *et al.* (2004) Bacterial flagellin is a dominant antigen in Crohn disease. *J. Clin. Invest* 113, 1296-1306

45 Kobayashi,K. *et al.* (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416, 194-199

46 Wolfert,M.A. *et al.* (2002) The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan. *J. Biol. Chem.* 277, 39179-39186

47 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

48 Endres,S. *et al.* (1988) Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin. Immunol. Immunopathol.* 49, 424-438

49 Takeuchi,O. *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11, 443-451

50 Kullberg,B.J. *et al.* (1993) Recombinant interferon-gamma enhances resistance to acute disseminated Candida albicans infection in mice. *J. Infect. Dis.* 168, 436-443

51 Drenth,J.P. *et al.* (1995) Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNFalpha and IL-1 beta production. *J. Appl. Physiol* 79, 1497-1503

52 Netea,M.G. *et al.* (1996) Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. *J. Clin. Invest* 97, 1366-1372

53 Lien,E. *et al.* (2000) Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest* 105, 497-504

Chapter 8

Engagement of Nod2 has a dual effect on pro IL-1β mRNA transcription and secretion of bioactive IL-1β

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Abstract

Synthesis and release of pro-inflammatory cytokines, such as IL-1β, play a crucial role in the intestinal inflammation that characterizes Crohn's disease. Mutations in the nucleotide oligomerization domain 2 (Nod2) gene are associated with an increased risk of Crohn's disease. Although it is known that Nod2 mediates cytokine responses to muramyl dipeptide (MDP), it is yet unclear whether Nod2 stimulation mediates only transcription of pro- IL-1β mRNA, or whether Nod2 is also involved in the activation of caspase-1 and release of active IL-1β. By investigating the response of MNC from Crohn's disease patients homozygous for the 3020insC Nod2 mutation, we were able to show that Nod2 signaling after stimulation with MDP has a dual effect by activating pro IL-1β mRNA transcription and inducing release of bioactive IL-1β. Because Nod2 engagement amplifies TLR stimulation, we investigated whether activation of caspase-1 by MDP is involved in the Nod2/TLR synergism. The synergy in IL-1β production between Nod2 and TLR is mediated at post-translational level in a caspase-1-dependent manner, which indirectly suggests that Nod2 also induces caspase-1 activation. In contrast, the synergy in TNFα production after stimulation with MDP and LPS is induced at transcriptional level. This demonstrates that both caspase-1-dependent and -independent mechanisms are involved in the synergy between Nod2 and TLR.

Introduction

NOD-like receptors (NLR) are intracellular receptors for bacterial peptidoglycans, which complement the recognition of pathogen-associated molecular patterns (PAMP) by membrane-bound TLR [1,2]. Nucleotide oligomerization domain 2 (Nod2) is a member of the NACHT-LRR (NLR) receptor family, which recognizes muramyl dipeptide (MDP), the minimal motif of peptidoglycan of both Gram-positive and Gram-negative bacteria [3]. Mutations in the Nod2 gene are associated with Crohn's disease [4,5], but how Nod2 exactly acts in the pathogenesis of this auto-inflammatory disease is unclear [6-8]. Therefore, a better understanding of the intracellular events induced by the interaction between Nod2 and peptidoglycan is crucial for both the insight into recognition of Gram-positive pathogens by the innate immune system, and for the pathogenesis of the inflammatory reactions in Crohn's disease.

Activation of human mononuclear cells (MNC) by MDP leads to production of pro-inflammatory cytokines, especially IL-1β [7,9]. IL-1β is produced as pro- IL-1β a 31-34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to the bioactive 17-kDa IL-1β [10]. This is followed by IL-1β excretion in microvesicles into the extracellular environment [11]. Apparently, MDP is capable of inducing all three steps, but it is unclear whether Nod2 alone or other receptors are involved in one or more of these steps of IL-1β production. It has been proposed that several of the NLR family members are able to recognize MDP, most notably Nod2, NALP3, and NALP1, and that they execute different functions necessary for cytokine production. In this concept, recognition of MDP by Nod2 would mainly activate NF-κB and thereby gene transcription of pro- IL-1β, whereas NALP3/NALP1-mediated recognition of MDP leads to caspase-1 activation and the subsequent release of the active IL-1β form [12-14]. However, there are controversies regarding this mechanism of IL-1β production by MDP. The caspase-recruitment domain (CARD) of Nod2 interacts with the serine/tyrosine kinase RIP2, leading to NF-κB translocation and transcription of mRNA for pro-inflammatory cytokines [15-17]. However, it is also known that RIP2 interacts with the CARD domain of caspase-1 [18,19]. Thus, one could envisage that Nod2 engagement by MDP could activate caspase-1 and lead to the release of mature IL-1β, without the need for a secondary interaction with NALP3/NALP1.

In the present study, we aimed to assess whether recognition of MDP by Nod2 is important for both the induction of pro-inflammatory cytokines gene transcription, as well as for the activation of caspase-1 and IL-1β release, by comparing the response of MNC from patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs) with the response of MNC isolated from individuals with wild-type Nod2 allele (Nod2wt). In addition, because MDP is able to amplify the TLR stimulation through a Nod2-dependent pathway [9,20], we also investigated whether activation of caspase-1 by MDP is involved in the Nod2/TLR synergism.

Results

MDP induces Nod2-dependent transcription of cytokine genes

In MNC of Crohn's disease patients with a homozygous 3020insC mutation (Nod2fs), the cytokine production after stimulation with MDP is completely abolished, whereas the cytokine production after stimulation with the TLR4 ligand LPS is normal compared to healthy volunteers (HC) and Crohn's disease patients bearing the wild-type allele (Nod2wt) (Fig. 1A). This impaired cytokine response to MDP is caused by a transcriptional defect, since MDP was found to increase mRNA in MNC of healthy volunteers and Crohn's disease patients without Nod2 mutations of IL-1β and TNFα, but not in patients homozygous for the 3020insC mutation (Fig. 1B).

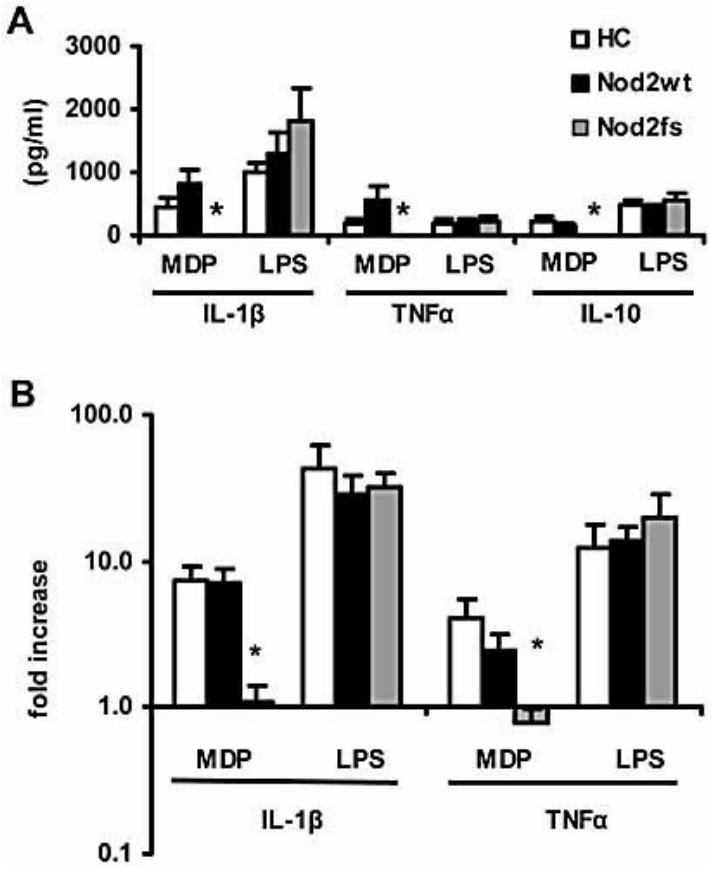

Figure 1. MDP induces Nod2 dependent cytokine production and transcription of pro-IL-1β and TNFα in MNC. MNC of five healthy controls (HC), five Crohn's patients without Nod2 mutations (Nod2wt) and four patients homozygous for the 3020insC mutation (Nod2fs) were stimulated with LPS (10 ng/mL) or MDP (100 nM). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37ºC (A). Quantitative measurement of mRNA levels of IL-1β and TNFα was performed by real-time PCR and expressed as fold increase compared to unstimulated cells. Cells were lysed in RNAzolB after 4-h incubation at 37ºC. (B). Data are presented as means ± SEM and compared by Mann-Whitney U test (*p <0.05).

Induction of IL-1β by MDP is caspase-1 dependent

Various bacterial stimuli can induce mature IL-1β production, which requires both transcription of pro- IL-1β mRNA and post-translational processing by caspase-1. The IL-1β production in MNC after stimulation with MDP was blocked with the caspase-1 inhibitor (ICE-i) Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (YVAD) (Fig. 2A). This indicates that the post-translational processing of pro- IL-1β by caspase-1 is important for the IL-1β production by MDP. Inhibition of the IL-1R with IL-1Ra did not block the IL-1β production (Fig. 2A). Thus, an autocrine feedback of IL-1β inducing IL-1β, as shown previously using LPS as a stimulus [21], does not seem to play a major role in MDP-induced IL-1β.

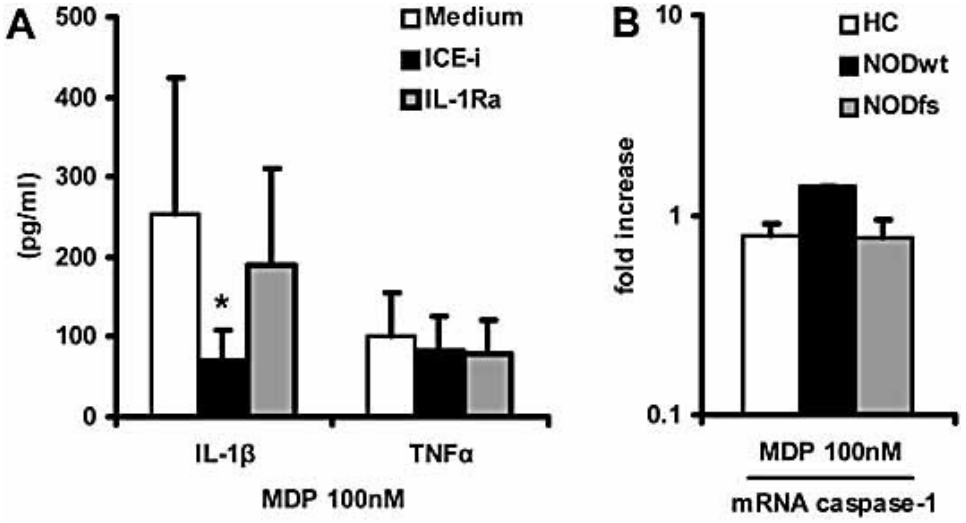

Figure 2. MDP activates caspase-1 but does not enhance transcription of caspase-1 mRNA. Stimulation of MNC of five healthy volunteers with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 μM) or the IL-1-receptor with IL-1Ra (10 μg/mL). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37ºC (A). MNC of four healthy controls (HC), four Crohn's patients without Nod2 mutations (Nod2wt) and four patients homozygous for the 3020insC mutation (Nod2fs) were stimulated with MDP (100 nM). After 4-h incubation at 37ºC, cells were lysed in RNAzolB. Quantitative measurement of mRNA levels of caspase-1 was performed by real-time PCR and expressed as fold increase compared to unstimulated cells (B).

During the exposure to MDP, caspase-1 mRNA levels did not increase compared to unstimulated cells, as shown in Fig. 2B. Since the caspase-1 activity is not regulated at transcriptional level, the regulation of caspase-1 activation is likely post-translational by enzymatic cleavage of pro-caspase-1 [22].

The synergism between Nod2 and TLR for IL-1β is caspase-1 dependent

Stimulation of cells with MDP and the TLR4 ligand LPS induces cytokines in a synergistic fashion [9], [20]. Because MDP can activate caspase-1 and induce mature IL-1β,

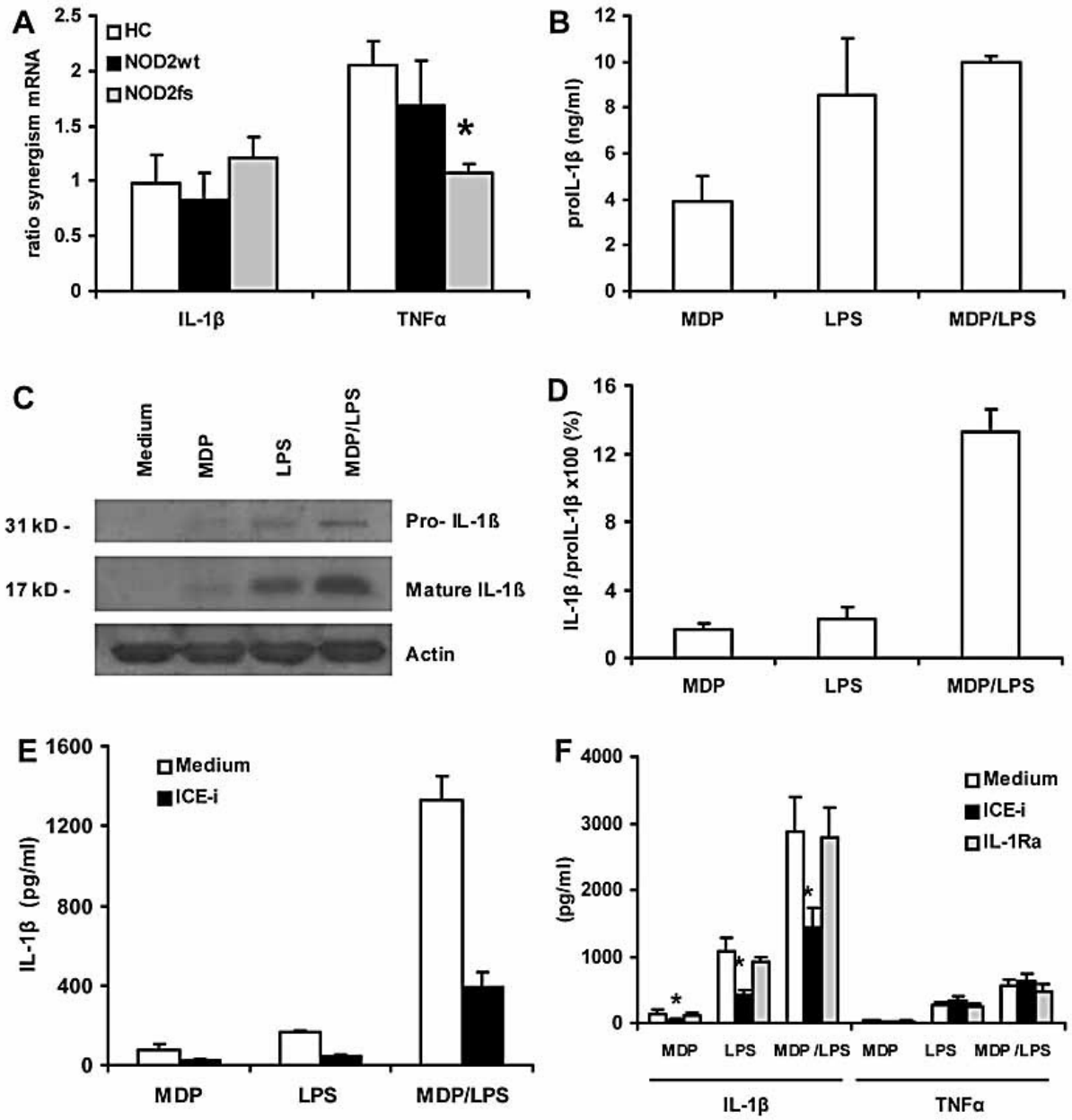

Figure 3. The synergism between TLR and Nod2 for the release of IL-1β is exerted at post-transcriptional level. MNC from four healthy volunteers were stimulated with MDP (100 nM), LPS (10 ng/mL) or a combination of MDP and LPS. After 4-h incubation at 37ºC, cells were lysed in RNAzolB. Quantitative measurement of mRNA levels of IL-1β and TNFα was performed by real-time PCR and expressed as fold increase compared to LPS-treated cells (A). The proIL-1β production after 4-h stimulation was assessed by a specific ELISA (B) or Western blots (C). Similarly, IL-1β concentrations were assessed by an ELISA (E). The percentages of mature IL-1β secreted from the cells when they were stimulated with MDP, LPS or the combination MDP/LPS are presented in (D). Stimulation of MNC of nine healthy volunteers with LPS (10 ng/mL) in combination with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 µM) or the IL-1R with IL-1Ra (10 µg/mL). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37ºC (F). Data presented as means ± SEM and compared by Mann-Whitney U test (*p <0.05).

investigated whether caspase-1 is important for this synergy. The induction of IL-1β mRNA in MNC of HC, Nod2wt and Nod2fs does not increase after stimulation with MDP and LPS compared to LPS alone (Fig. 3A). In addition, there is no synergy in the production of pro-IL-1β production, as assessed both by a specific pro-IL-1β ELISA (Fig. 3B) or Western blots (Fig. 3C). However, mature IL-1β is secreted in a synergistic way (Fig. 3E). The increased ratio between intracellular pro IL-1β and secreted IL-1β indicates that the synergistic production of IL-1β after stimulation with MDP and LPS is established either by extra cleaving of pro-IL-1β or/and by increased release of bioactive IL-1β (Fig. 3D).
In support of the notion that processing of pro- IL-1β is the level at which the synergism between Nod2 and TLR takes place, when caspase-1 was inhibited with YVAD (ICE-i) the synergy declined, showing that caspase-1 is responsible for the synergy of IL-1β production after stimulation with MDP and LPS (Fig. 3F). In contrast to IL-1β, the induction of TNFα mRNA in MNC of HC and Nod2wt increases after stimulation with MDP and LPS compared to LPS alone in the same ratio as TNFα in the supernatant. In MNC of Nod2fs no increase of TNFα mRNA and TNFα is found (Fig. 3A). Inhibition of caspase-1 does not influence the TNFα production or synergy (Fig. 3F). Blocking the IL-1R with IL-1Ra has no effect on the synergistic production of IL-1β and TNFα, indicating that autocrine stimulation of IL-1R cannot explain the synergy (Fig. 3F). These data show that the synergy in TNFα production after stimulation with MDP and LPS is induced at transcriptional level, whereas the synergy in IL-1β production is regulated at post-translational level by activation of caspase-1.

Activation of caspase-1 and release of bioactive IL-1β by MDP is Nod2 dependent

To test whether MDP uses another receptor than Nod2 for the activation of caspase-1 (e.g. NALP3), and in the absence of a reliable caspase-1 p10 Western-blot methodology in human primary cells, we designed an indirect functional assay to evaluate this hypothesis. If MDP would activate capsase-1 through NALP3 or NALP1, one would expect that although MDP is unable by itself to induce IL-1β in cells from Nod2fs patients, due to its inability to activate transcription, it would still be able to amplify IL-1β production induced by a TLR ligand (Fig. 4). This would happen because of the intracellular pro IL-1β induction by the TLR agonist, while processing of IL-1β at the level of caspase-1 activation would be amplified by the MDP-NALP3/NALP1 interaction (Fig. 4A). However, the data presented in Fig. 4B strongly argue against this hypothesis. These data show clearly that MDP was unable to amplify IL-1β production when cells of Crohn's disease patients with Nod2fs were stimulated with LPS, suggesting that Nod2 is necessary for the caspase-1 activation by MDP.

In order to investigate the role of Nod2 for the release of active IL-1β from the cells, we have tested the effects of the Nod2-deficiency in an ATP/LPS stimulation assay. When ATP was added to LPS-primed cells, a significant increase in the IL-1β secreted from the cells bearing only the wild-type Nod2 allele was observed. In contrast, the ATP-dependent IL-1β release was severely impaired in the cells isolated from individuals homozygous for the Nod2fs mutation (Fig. 4C). IL-1β production after LPS stimulation alone was not different in Nod2wt and Nod2fs individuals (Fig. 4C).

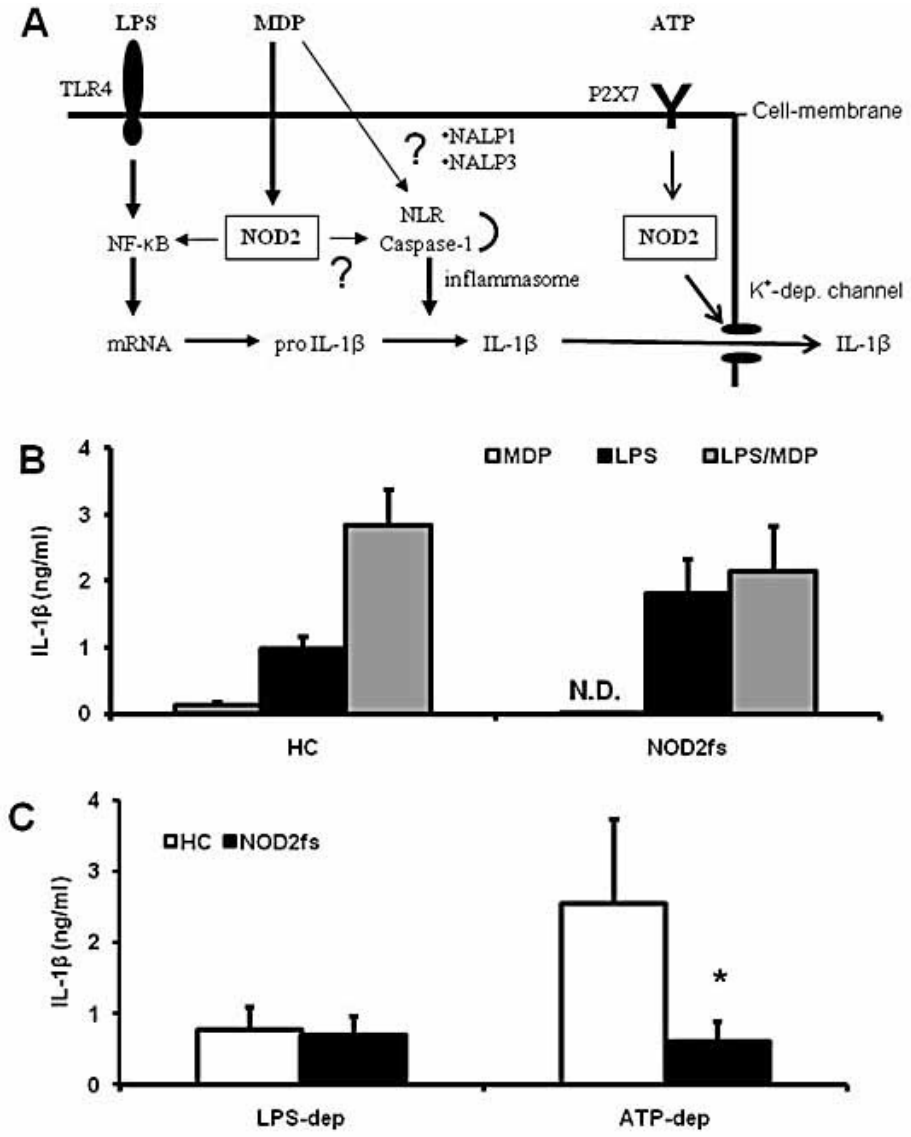

Figure 4. Activation of caspase-1 by MDP is Nod2 dependent. Putative scheme of activation of the inflammasome by MDP, in which the MDP/Nod2 interaction activates transcription, processing and release of IL-1β. A potential interaction of Nod2 with NALP3/NALP1 for the activation of the inflammasome is still unclear (A). Stimulation of MNC of four healthy controls (HC) and 4 patients homozygous for the 3020insC mutation (Nod2fs) with MDP (100 nM), LPS (10 ng/mL) or a combination of MDP and LPS. Cells were incubated for 24 h at 37ºC and IL-1β was measured in the supernatant by ELISA (B). The role of Nod2 for the release of IL-1β by MNC primed for 4 h with LPS (1 µg/mL), and after an additional incubation with ATP (1 mM) for 15 min (C). Data are presented as means ± SEM and compared by Mann-Whitney U test (*p <0.05).

Discussion

In this study, we show that Nod2 has dual effects on both pro IL-1β mRNA transcription and the release of bioactive IL-1β from the cells. In addition, indirect functional tests on the role of caspase-1 for the synergistic effects between Nod2 and TLR also suggest a role for Nod2 for the activation of caspase-1 by MDP, the active component of bacterial peptidoglycan.

The regulation and activation of caspase-1 and thereby the regulation of the production of mature IL-1β, followed by its secretion from the cells, is a rapid evolving field of research. The importance of understanding these crucial steps in IL-1β processing and release is stressed by the role of IL-1β in virtual all forms of inflammation and especially in auto-inflammatory diseases, such as familial Mediterranean fever (FMF), hyperimmunoglobulinemia D syndrome (HIDS) and Muckle-Wells syndrome [22]. Targeting IL-1β in these disorders has shown spectacular results [23-27], and targeting caspase-1 could lead to new treatment strategies. Some reports indicate that caspase-1 activity is partially regulated at transcriptional level [28]. However, our results show that stimulation with MDP did not result in an increase expression of caspase-1 mRNA. This is in line with the current opinion that caspase-1 activity is mainly regulated at post-translational level [29,30]. In this view, caspase-1 is activated by close proximity mechanism in a multimeric protein platform, called the inflammasome. It is unclear which pathway directly activates the inflammasome and how many different of these platforms exists. The most studied inflammasomes consist of NALP3/ASC/cardinal/caspase-1 and NALP1/ASC/caspase-1/caspase-5. NALP belong to the same protein family as NOD and CIITA, which is named the NLR (NACHT-LRR receptors or NOD-like receptors) family. All NLR have a NACHT domain that is involved in forming multimers. In addition, some members have a CARD used for interaction with other CARD-containing proteins, such as caspases. Finally, the LRR domain can interact with PAMP (e.g. MDP interaction with LRR of Nod2). An interesting hypothesis is that the interaction of a PAMP with the LRR of an NLR in the inflammasome can activate caspase-1. Indeed, Martinon et al. [12] proposed that MDP interacts with the LRR of NALP3 in cell lines and can activate the inflammasome in human macrophages. Similarly, a recent study has suggested that NALP-1 is also an MDP receptor activating caspase-1 [14].

In human MNC, we confirm the activation of the inflammasome by MDP, indicated by the release of mature IL-1β after stimulation with MDP. Importantly, indirect proof based on a Nod2/TLR stimulation assay suggests that activation of the caspase-1 seems to occur in a Nod2-dependent manner. If MDP would activate caspase-1 independently of Nod2, stimulation of cells lacking a functional Nod2, such as Nod2fs MNC, with LPS and MDP should result in an amplification of LPS-induced IL-1β production through MDP-NALP3/NALP1-dependent mechanisms. In Nod2-deficient cells, LPS induces pro-IL-1β and activation of caspase-1, and MDP should amplify caspase-1 activation and IL-1β release in a Nod2-independent, NALP3- or NALP1-dependent manner. However, the data presented here clearly show that this is not the case: no increase in IL-1β production was documented

when Nod2fs cells are stimulated with LPS and MDP at the same time. This suggests that Nod2 has a non-redundant function for caspase-1 activation by MDP (Fig. 4A). However, it cannot be excluded that Nod2 also requires the presence of NALP3 and/or NALP1 and collaborate with one or both of these molecules for the activation of caspase-1. A recent study by Pan and colleagues [31] showing that both Nod2 and NALP3 are necessary for IL-1β secretion by MDP in murine macrophages gives weight to this hypothesis. Unfortunately, the lack of a reliable Western blot assay for the activated caspase-1 p10 in human primary monocytes has precluded us to obtain direct evidence of the role of Nod2 for the caspase-1 activation. However, by using a well-established model of IL-1β release after LPS priming of cells, followed by stimulation with the K+-channel activator ATP, we were able to demonstrate an important role for Nod2 in the release of active IL-1β. This is in line with a recent study showing that NALP3, another member of the NLR family, binds ATP [32].

Some of the differences between our findings and the study of Martinon et al. [12] in terms of IL-1β induction by purified LPS could be explained by the use of different cell types: freshly MNC produce mature IL-1β after stimulation with LPS, whereas monocytes-derived macrophages do not. Furthermore, it has to be realized that data from cell lines and overexpression models are not always compatible with data obtained from freshly isolated human cells. In addition, differences exist between NALP3-/- mice and human cells [12,33,34], and between Nod2-/- mice and cells obtained from Crohn's disease patients homozygous for the Nod2fs allele [7,35].

The unique role of Nod2 as key receptor of MDP for the induction of NF-B-dependent transcription of mRNA of cytokines is underlined by showing that mRNA of IL-1β and TNFα in Nod2fs cells is not increased after stimulation with MDP. Subsequently, the production of the cytokines is abolished. Our quantitative data by real-time PCR, obtained in primary cells, are supported by the results obtained by other groups [36-38].

When MNC are stimulated with low dose MDP and the TLR4 ligand LPS, a remarkable synergy in cytokine production is apparent in MNC from individuals with an intact Nod2 [9,20]. A potential role for caspase-1 in the synergy between TLR4 and Nod2 could be envisaged, since MDP can activate caspase-1 and induce IL-1β, which is known to induce IL-1β production in an autocrine fashion through stimulation of the IL-1R [21]. In addition, IL-1β can induce the production of other cytokines, like IL-10 and IL-6 [39]. Indeed, the synergistic production of IL-1β by MDP and LPS is caspase-1dependent, and not regulated at transcriptional level. However, in line with other studies, we show that the mechanism of synergy for TNFα is exerted at a transcriptional level [40].

In conclusion, we demonstrate that Nod2 is non-redundant for both the production of IL-1β mRNA and the IL-1β release from the activated cells. In addition, indirect data supports a role for Nod2 in the activation of caspase-1 in human MNC after stimulation with MDP. Furthermore, caspase-1-dependent mechanisms are responsible for the synergistic effect on IL-1β production between Nod2 and TLR. Currently, it is unclear whether Nod2 is part of an

inflammasome protein complex or it directly activates caspase-1. Further investigation is needed to unravel the complete extension of the role of Nod2, but the picture emerges that Nod2 is the key receptor for MDP and executes its function at different levels in the pathways of cytokine production.

Materials and methods

Reagents

Synthetic Pam3Cys was purchased from EMC Microcollections (Tubingen, Germany). LPS (Escherichia coli serotype 055:B5) was purchased from Sigma (St. Louis, MO) and an extra purification step was performed as previously described [41]. The purified LPS was tested in TLR4-/- mice for the presence of contaminants and it did not have any TLR4-independent activity. Synthetic MDP was obtained from Sigma. The reversible caspase-1 inhibitor (ICE-i) Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (YVAD) was purchased from Alexis Biochemicals (San Diego, CA) and solubilized in DMSO at 10 mg/ml. The ICE-i was diluted to the desired concentration in RPMI.

Genotyping of Nod2 variants

Blood was collected from 154 patients with Crohn's disease and 10 healthy volunteers. PCR amplification of Nod2 gene fragments containing the polymorphic site 3020insC was performed in 50-L reaction volumes containing 100-200 ng genomic DNA as previously described [42]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems).

Seven patients with Crohn's disease were found homozygous for the 3020insC mutation, and four of them were further investigated in the cytokine studies. As control groups, four patients with Crohn's disease and four healthy volunteers homozygous for the wild-type Nod2 allele were included.

Isolation of MNC and stimulation of cytokine production

After obtaining informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 mL EDTA tubes (Monoject). Isolation of MNC was performed as described elsewhere [43], with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and suspended in culture medium (RPMI 1640) supplemented with gentamicin 10 µg/ml, L-glutamine 10 mM and pyruvate 10 mM. The cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to 5×10^6 cells/ml. MNC (5×10^5) in a 100-L volume were added to round-bottom 96-wells plates (Greiner) and incubated with either 100 L of culture medium (negative control), or the various stimuli: LPS (1 ng/ml), MDP (10 nM), or combinations of MDP and LPS at various concentrations. In separate experiments, inhibitors (ICE-i, 20 µM or IL-1Ra, 10 µg/ml) were added 10 min before stimulation. The stimuli were checked for the contamination with LPS in the LAL assay and found to be negative. After 24 h, the supernatants were collected and stored at -70°C until assayed.

To investigate the role of Nod2 for the release of IL-1β, PBMC were initially stimulated for 4 h with LPS (1 µg/mL). After 4 h, supernatants were collected and medium containing 1 mM ATP was added to the cells for another 15 min. The LPS-dependent IL-1β production during the first 4 h and the ATP-dependent IL-1β secretion after the additional 15 min was assessed in the supernatant.

Cytokine measurements
Human TNFα concentrations were determined by specific ELISA [44]. IL-1β, proIL-1 and IL-10 were measured by commercial ELISA kits (R&D Systems, and Pelikine Compact, Sanquin), according to the instructions of the manufacturer.

Quantitative PCR
MNC were stimulated as described above, after 4 h the supernatant was removed and the cells resuspended in 200 µL RNAzolB RNA isolation solvent (Campro Scientific) and frozen at -80°C for storage. mRNA was isolated following the manufacturer's protocol. The amount and quality of mRNA were determent by spectrophotometry and analyzed by agarose gel electrophoresis for DNA contamination. cDNA was synthesized from 1000 ng of total RNA using SuperScript^{tm} Reverse Transcriptase (Invitrogen; 18064-014).

Relative mRNA levels were determined using the Bio-Rad iCycler and SYBR Green method (Invitrogen; S7563) [45]. The following primers were used: IL-1β forward (5-TGGCCCAGGCGTCAGA-3), IL-1β reverse (5-GGTTTGCTACAACATGGGCTACA-3); TNFα forward (5-GCCCTAAACAGATGAAGTGCTC-3), TNFα reverse (5-GAACCAGCATCTTCCTCAG-3); B2M forward (5-ATGAGTATGCCTGCCGTGTG-3), B2M reverse (5-CCAAATGCGGCATCTTCAAAC-3) (Biolegio, Malden, The Netherlands). Mean relative mRNA expression from at least two replicate measurements was calculated using Bio-Rad iCycler IQ software. Values are expressed as fold increase to mRNA levels of unstimulated cells.

Western blots for pro- IL-1β and IL-1β
Human MNC were stimulated as described above. After 4-h stimulation, supernatant was removed and cells were lysed in 25 L of ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.1% SDS and protease inhibitors (SigmaFast, Sigma). Lysates of 4 × 106 cells were pooled. Samples were taken up in 25 µL sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% -mercaptoethanol, 10% glycerol and 0.5 mg/mL of bromophenol blue), were separated by SDS-PAGE and were blotted onto polyvinyldifluoride membranes (Millipore). Membranes were blocked in 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dry milk and were incubated overnight with antibody against cleaved IL-1β (Cell Signaling) in 1% BSA and 0.1% Tween-20 in Tris-buffered saline. Horseradish peroxidase-conjungated secondary antibodies were visualized with Lumilite plus (Boehringer-Mannheim).

Statistical analysis
All experiments were performed at least in duplicate with blood obtained from four Crohn's disease patients bearing the Nod2fs mutation, four Crohn's disease patients with the wild-type Nod2 genotype, as well as four healthy volunteers. Synergy was expressed as ratio of cytokine response of ligand in combination with MDP divided by the sum of cytokine responses obtained with each ligand alone. The differences in cytokine production between groups were analyzed by Mann-Whitney U test or Wilcoxon, and where appropriate by Kruskal-Wallis ANOVA test. For all other comparisons the Student's t-test was used. The level of significance between groups was set at $p < 0.05$. The data are given as means ± SEM.

Reference List

1 Inohara,N. and Nunez,G. (2003) NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* 3, 371-382

2 Ting,J.P. and Davis,B.K. (2005) CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu. Rev. Immunol.* 23, 387-414

3 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

4 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

5 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

6 Eckmann,L. and Karin,M. (2005) NOD2 and Crohn's disease: loss or gain of function? *Immunity.* 22, 661-667

7 Netea,M.G. *et al.* (2005) NOD2 3020insC mutation and the pathogenesis of Crohn's disease: impaired IL-1beta production points to a loss-of-function phenotype. *Neth. J. Med.* 63, 305-308

8 Watanabe,T. *et al.* (2005) NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease. *Gut* 54, 1515-1518

9 van Heel,D.A. *et al.* (2005) Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 365, 1794-1796

10 Dinarello,C.A. (1998) Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. *Ann. N. Y. Acad. Sci.* 856, 1-11

11 Mackenzie,A. *et al.* (2001) Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity.* 15, 825-835

12 Martinon,F. *et al.* (2004) Identification of Bacterial Muramyl Dipeptide as Activator of the NALP3/Cryopyrin Inflammasome. *Curr. Biol.* 14, 1929-1934

13 Agostini,L. *et al.* (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity.* 20, 319-325

14 Faustin,B. *et al.* (2007) Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol. Cell* 25, 713-724

15 Kobayashi,K. *et al.* (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416, 194-199

16 Inohara,N. *et al.* (2000) An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* 275, 27823-27831

17 Abbott,D.W. *et al.* (2004) The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr. Biol.* 14, 2217-2227

18 Thome,M. *et al.* (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* 8, 885-888

19 Yoo,N.J. *et al.* (2002) Nod1, a CARD protein, enhances pro-interleukin-1beta processing through the interaction with pro-caspase-1. *Biochem. Biophys. Res. Commun.* 299, 652-658

20 Netea,M.G. *et al.* (2005) Nucleotide-Binding Oligomerization Domain-2 Modulates Specific TLR Pathways for the Induction of Cytokine Release. *J. Immunol.* 174, 6518-6523

21 Schindler,R. *et al.* (1990) IL-1 induces IL-1. IV. IFN-gamma suppresses IL-1 but not lipopolysaccharide-induced transcription of IL-1. *J. Immunol.* 144, 2216-2222

22 Dinarello,C.A. (1996) Biologic basis for interleukin-1 in disease. *Blood* 87, 2095-2147

23 Bodar,E.J. *et al.* (2005) Effect of etanercept and anakinra on inflammatory attacks in the hyper-IgD syndrome: introducing a vaccination provocation model. *Neth. J. Med.* 63, 260-264

24 Lovell,D.J. *et al.* (2005) Interleukin-1 blockade by anakinra improves clinical symptoms in patients with neonatal-onset multisystem inflammatory disease. *Arthritis Rheum.* 52, 1283-1286

25 Hawkins,P.N. *et al.* (2003) Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N. Engl. J. Med.* 348, 2583-2584

26 Hoffman,H.M. *et al.* (2004) Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 364, 1779-1785

27 Simon,A. *et al.* (2004) Beneficial response to interleukin 1 receptor antagonist in traps. *Am. J. Med.* 117, 208-210

28 Joshi,V.D. *et al.* (2002) Role of caspase 1 in murine antibacterial host defenses and lethal endotoxemia. *Infect. Immun.* 70, 6896-6903

29 Boatright,K.M. and Salvesen,G.S. (2003) Caspase activation. *Biochem. Soc. Symp.*, 233-242

30 Martinon,F. and Tschopp,J. (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117, 561-574

31 Pan,Q. *et al.* (2007) MDP-induced interleukin-1{beta} processing requires Nod2 and CIAS1/NALP3. *J. Leukoc. Biol.*

32 Duncan,J.A. *et al.* (2007) Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc. Natl. Acad. Sci. U. S. A* 104, 8041-8046

33 Mariathasan,S. *et al.* (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440, 228-232

34 Kanneganti,T.D. *et al.* (2006) Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440, 233-236

35 Maeda,S. *et al.* (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307, 734-738

36 Uehara,A. *et al.* (2005) Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a. *Cell Microbiol.* 7, 53-61

37 Ogura,Y. *et al.* (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J. Biol. Chem.* 276, 4812-4818

38 Li,J. *et al.* (2004) Regulation of IL-8 and IL-1beta expression in Crohn's disease associated NOD2/CARD15 mutations. *Hum. Mol. Genet.* 13, 1715-1725

39 Dinarello,C.A. (1998) Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *Int. Rev. Immunol.* 16, 457-499

40 Wolfert,M.A. *et al.* (2002) The origin of the synergistic effect of muramyl dipeptide with endotoxin and peptidoglycan. *J. Biol. Chem.* 277, 39179-39186

41 Hirschfeld,M. *et al.* (2001) Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun.* 69, 1477-1482

42 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

43 Endres,S. *et al.* (1988) Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin. Immunol. Immunopathol.* 49, 424-438

44 Grebenchtchikov,N. *et al.* (2005) Development of a sensitive ELISA for the quantification of human tumour necrosis factor-alpha using 4 polyclonal antibodies. *Eur. Cytokine Netw.* 16, 215-222

45 Arya,M. *et al.* (2005) Basic principles of real-time quantitative PCR. *Expert. Rev. Mol. Diagn.* 5, 209-219

Chapter 9

Nod2 and Toll-Like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*

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Abstract

Infection with *Mycobacterium tuberculosis* is one of the leading causes of death worldwide. Recognition of *M. tuberculosis* by pattern recognition receptors is crucial for activation of both innate and adaptive immune responses. In the present study, we demonstrate that nucleotide-binding oligomerization domain 2 (Nod2) and Toll-like receptors (TLRs) are two nonredundant recognition mechanisms of *M. tuberculosis*. CHO cell lines transfected with human TLR2 or TLR4 were responsive to *M. tuberculosis*. TLR2 knock-out mice displayed more than 50% defective cytokine production after stimulation with mycobacteria, whereas TLR4-defective mice also released 30% less cytokines compared to controls. Similarly, HEK293T cells transfected with Nod2 responded to stimulation with *M. tuberculosis*. The important role of Nod2 for the recognition of *M. tuberculosis* was demonstrated in mononuclear cells of individuals homozygous for the 3020insC Nod2 mutation, who showed an 80% defective cytokine response after stimulation with *M. tuberculosis*. Finally, the mycobacterial TLR2 ligand 19-kDa lipoprotein and the Nod2 ligand muramyl dipeptide synergized for the induction of cytokines, and this synergism was lost in cells defective in either TLR2 or Nod2. Together, these results demonstrate that Nod2 and TLR pathways are nonredundant recognition mechanisms of *M. tuberculosis* that synergize for the induction of proinflammatory cytokines.

Synopsis

Tuberculosis is one of the most prevalent infections worldwide, with 2 billion people believed to be infected, and 2 million deaths each year. In addition to representing a major health care problem in developing countries, concern is also growing about the increased incidence of tuberculosis in developed countries, especially in immunocompromised patients such as those with AIDS, transplantation, and immunosuppressive therapy. The present study describes the pathways that enable leukocytes to recognize *M. tuberculosis*, and demonstrates for the first time that Nod2, member of a new class of intracellular receptors, is an independent recognition mechanism for mycobacteria. Nod2 acts together with the earlier-described Toll-like receptors for the activation of host defenses during the encounter of leukocytes with *M. tuberculosis*. Understanding the mechanisms through which the cells of the immune system recognize *M. tuberculosis* can be an important step in designing new therapeutic approaches, as well as improving the limited success of current vaccination strategies.

Introduction

Worldwide, 2 billion people are currently believed to be infected with *Mycobacterium tuberculosis*, with an estimated death toll of 2 million patients each year [1]. *M. tuberculosis* is an intracellular pathogen capable of infecting and surviving within the host's mononuclear cells (MNCs), and a coordinated response of the innate and adaptive immune systems is required for an effective host defense. This involves sequestration of the microorganism in macrophages within organized granulomas, and elimination of the pathogen through a combination of killing mechanisms and apoptosis of host macrophages [2]. These responses are coordinated by T helper 1-type proinflammatory cytokines, which are synthesized by phagocytes upon recognition of pathogen-associated molecular patterns of mycobacteria by pattern recognition receptors (PRRs).
Toll-like receptors (TLRs) are believed to be an important pattern recognition system of *M. tuberculosis*. A soluble, heat-stable mycobacterial fraction was initially reported to signal through TLR2, whereas heat-labile components associated with the cell wall were reported to signal through TLR4 [3]. Later, several components of mycobacteria were identified as being responsible for TLR2-dependent activation: the 19-kDa lipoprotein [4], lipomannan [5], phosphatidyl-myo-inositol mannoside [6], but not mannosyl-caped lipoarabinomannan from virulent *M. tuberculosis*, which mainly has anti-inflammatory effects through its interaction with the mannose receptor and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin, also termed DC-SIGN [7,8]. Despite an abundance of in vitro data regarding the recognition of mycobacterial structures by TLR2 and TLR4, knock-out mice deficient for these receptors display remarkably little enhanced susceptibility to infection with *M. tuberculosis*. TLR4−/− mice showed variable responses to the challenge with *M. tuberculosis*, with either normal resistance to infection [9,10] or chronic pneumonia and increased mortality [11,12]. In one study, TLR2−/− mice had a decreased clearance of the bacteria and developed chronic pneumonia when infected with a low dose of microorganisms [13], whereas in other studies only minor effects have been found [14,15]. The role of TLRs in the recognition of mycobacteria was further highlighted in mice deficient for MyD88 (an adaptor molecule shared by almost all the receptors of the TLR family), which are highly susceptible to *M. tuberculosis* infection [16].

Nucleotide-binding oligomerization domain 2 (Nod2), initially described as a susceptibility gene for Crohn's disease [17,18], is an intracellular protein containing leucine-rich repeats (LRRs) similar to those found in TLRs. Nod2 is the PRR responsible for recognition of bacterial peptidoglycans from both Gram-positive and Gram-negative bacteria, through its interaction with muramyl dipeptide (MDP) [19]. In contrast, Nod1 recognizes peptidoglycans of Gram-negative bacteria only [20]. The intracellular localization of both Nod2 and *M. tuberculosis*, the cell wall of which contains peptidoglycans, makes Nod2 a highly suitable candidate for the recognition of mycobacteria. Our results support the hypothesis that TLRs and Nod2 represent two nonredundant recognition systems of *M. tuberculosis*, and that an efficient activation of innate immunity requires both classes of receptors.

Results

The role of TLR2 and TLR4 in the recognition of M. tuberculosis

TLR2 and TLR4 have been suggested to recognize bacterial structures of *M. tuberculosis* [21]. Indeed, sonicated *M. tuberculosis* strongly activated a Chinese hamster ovary fibroblast (CHO) cell line co-transfected with human TLR2 and CD14, whereas cells transfected with CD14 alone or a combination of CD14 and TLR4 displayed no signaling upon activation with the sonicated mycobacterial preparation (Figure 1A). However, when cells were stimulated with a preparation of whole mycobacteria, both TLR2 and TLR4-transfected cells were activated, although TLR2 activation was stronger (Figure 1A).

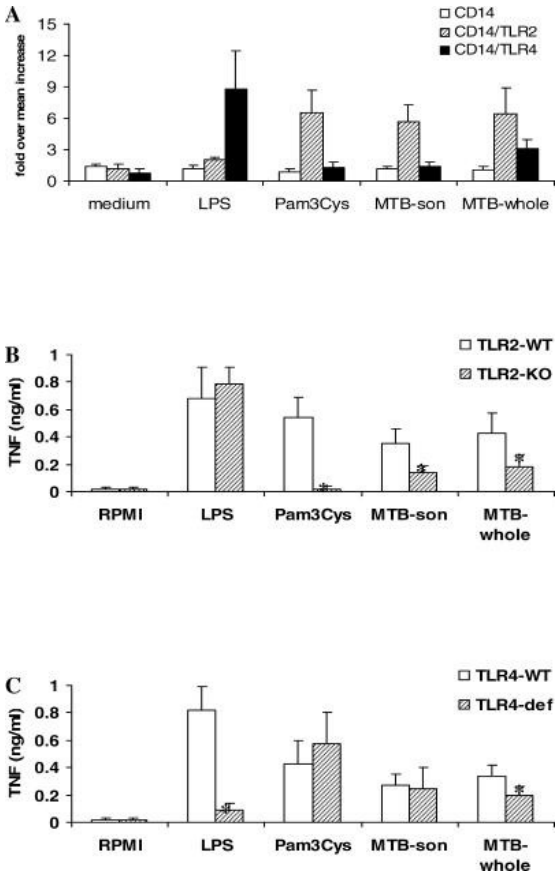

Figure 1. TLR2 and TLR4 are partially responsible for induction of cytokine production by *M. tuberculosis.* (A) CHO cells co-transfected with CD14 and TLR2 (CD14/TLR2, hatched bars) induced potent expression of CD25 on the cell membrane as measured by FACS analysis, after stimulation with both a sonicated *M. tuberculosis* (MTB-son, 1 μg/ml) or whole mycobacteria (MTB-whole, 1 × 10^7/ml). In contrast, cells transfected with CD14 and TLR4 (CD14/TLR4, black bars) were only moderately activated by whole M. tuberculosis, but not by the sonicated material. LPS (1 μg/ml) and Pam3Cys (5 μg/ml) served as control stimuli for TLR4 and TLR2, respectively. As controls, cells transfected only with CD14 were used (white bars). (B and C) Stimulation of TNF production by *M. tuberculosis* in peritoneal macrophages of mice deficient for TLR2 (TLR2-KO) (B) or TLR4 (TLR4-def) (C). Groups of five mice were stimulated with the indicated reagents, and the experiment was repeated twice. Medium-stimulated cells resulted in cytokine concentrations below detection limit. Data are presented as mean ± SD (n = 5; *$p < 0.05$).

In line with these data, macrophages isolated from TLR2−/− mice displayed a 50%–75% reduction in tumor necrosis factor (TNF) production after stimulation with both *M. tuberculosis* preparations (Figure 1B), whereas TLR4-deficient macrophages showed a 30%–40% reduction of TNF release only when stimulated with the whole mycobacteria (Figure 1C). To confirm the role of TLR4 in the stimulation of cytokines by *M. tuberculosis*, we stimulated human MNCs with the whole mycobacterial preparation in the absence or presence of 10 μg/ml of a blocking anti-TLR4 antibody. TLR4 blockade completely inhibited lipopolysaccharide (LPS)-induced TNF secretion, and reduced *M. tuberculosis*-induced TNF secretion from 0.9 ± 0.2 to 0.5 ± 0.2 ng/ml ($p < 0.05$). These data confirm the role played by TLR2 and TLR4 in the recognition of *M. tuberculosis*; however, the significant remaining production of cytokines induced by *M. tuberculosis* in TLR2−/− or TLR4-defective mice points to the presence of additional signaling pathway(s) for cytokine induction.

The role of intracellular recognition systems in the recognition of M. tuberculosis

The role of internalization in cytokine induction by *M. tuberculosis* was assessed by blocking it with cytochalasin B, an inhibitor of actin polymerization. Blocking internalization of *M. tuberculosis* partially inhibited *M. tuberculosis*-induced cytokine release in freshly isolated human MNCs (Figure 2). In contrast, cytochalasin B increased the zymosan-induced cytokine production in these MNCs, which likely results from prolonged stimulation of receptors at the cell surface by zymosan. The difference in the effect of cytochalasin B on *M. tuberculosis*- or zymosan-induced cytokines strongly suggests that internalization of M. tuberculosis is important for the induction of cytokine production through intracellular receptors.

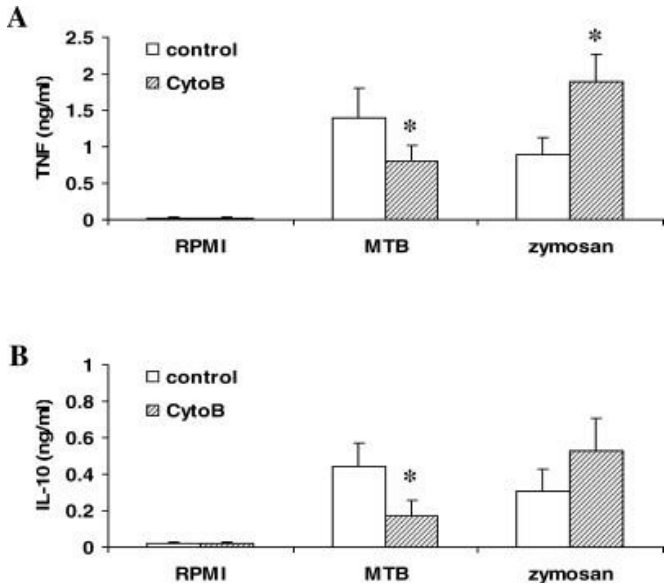

Figure 2. Blockade of internalization of M. tuberculosis impairs recognition and cytokine production. Blockade of *M. tuberculosis* internalization by cytochalasin B (20 μg/ml) impairs TNF (A) and IL-10 (B) stimulation in human MNCs stimulated with sonicated *M. tuberculosis* (1×10^6 microorganisms/ml), but not zymosan (1 μg/ml). Data are presented as mean ± SD ($n = 5$; $^*p < 0.05$).

The role of Nod1 and Nod2 in the recognition of M. tuberculosis

Next, human embryonic kidney 293T cells (HEK293Ts) were transfected with either Nod1 or Nod2 expression vectors, and the ability of *M. tuberculosis* sonicates to activate these pathogen recognition receptors was followed by monitoring the level of Nod-dependent activation of a nuclear factor κB (NF-κB)-driven luciferase reporter gene. Strikingly, *M. tuberculosis* sonicates efficiently stimulated Nod2, while Nod1-transfected cells responded only modestly to the bacteria (Figure 3).

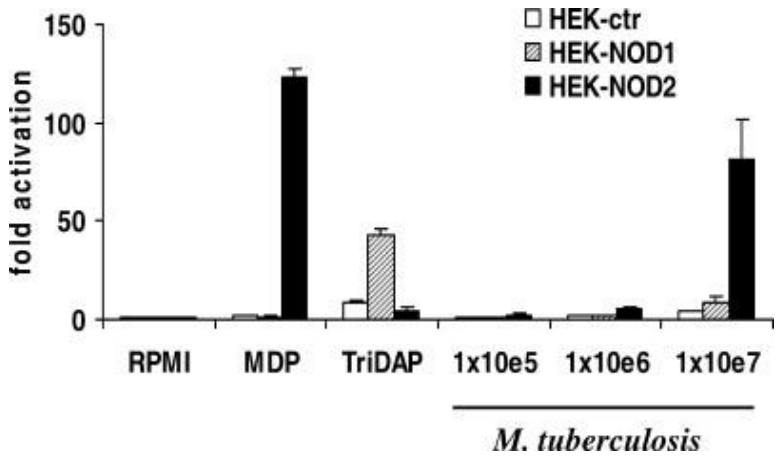

Figure 3. Stimulation of HEK cells transfected with Nod1 or Nod2. HEKs transfected with Nod2 were strongly activated by whole *M. tuberculosis*, in contrast to cells transfected with Nod1, which were only weakly activated by *M. tuberculosis*. MDP (100 nM) and TriDAP (100 nM) served as control stimuli for Nod2 and Nod1, respectively. Data are presented as fold increase over unstimulated cells (mean ± SD).

To investigate further the role of Nod1 and Nod2 in the recognition of *M. tuberculosis*, we stimulated peritoneal macrophages from Nod1−/− or Nod2−/− mice. We observed that peritoneal macrophages from Nod2−/− mice produced significantly less TNF than did control cells, supporting a role of Nod2 in the recognition of *M. tuberculosis* (Figure 4). In contrast, macrophages from Nod1−/− mice responded normally to *M. tuberculosis* (Figure 4), although as a control, their response to the Nod1 ligand FK156 was abrogated (unpublished data). Together, these results strongly support the notion that Nod2 represents an intracellular recognition system for *M. tuberculosis*.

Stimulation of cells from patients with defective Nod2 recognition

In line with the hypothesis that Nod2 is involved in the recognition of mycobacteria, MNCs isolated from patients homozygous for the 3020insC mutation synthesized 65%–80% less cytokines after stimulation with *M. tuberculosis* than did Crohn's disease patients heterozygous for the mutation or patients and volunteers homozygous for the wild-type variant (Figure 5A). This demonstrates that Nod2 is critical for the recognition of *M. tuberculosis* cell wall. In control experiments, cells from patients homozygous for the 3020insC mutation were defective in their response to MDP, but not LPS (Figure 5B), as previously described [22].

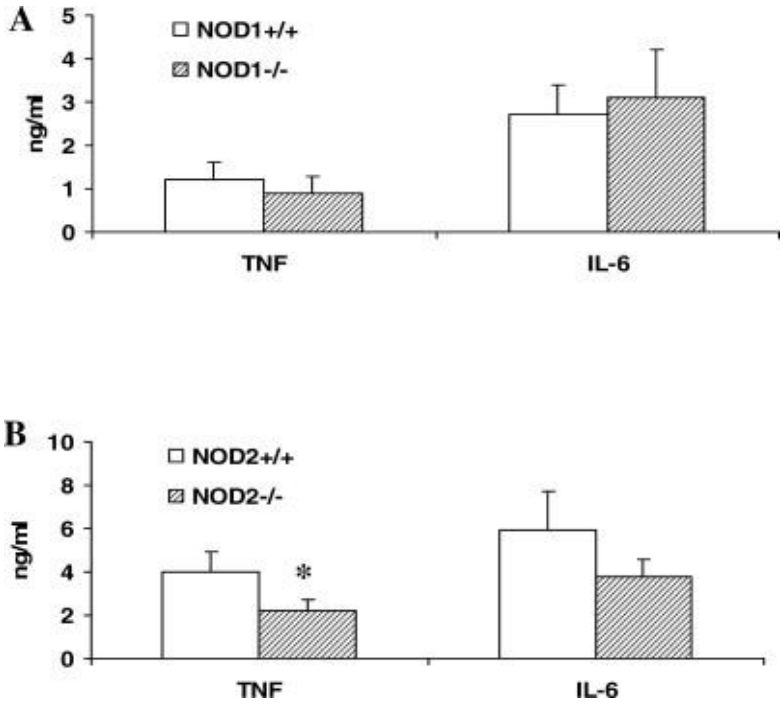

Figure 4. Stimulation of cells from mice deficient in Nod1 or Nod2. Stimulation of cytokine production by sonicated *M. tuberculosis* in peritoneal macrophages of mice deficient for either Nod1 (A) or Nod2 (B). Groups of four mice were stimulated; medium-stimulated cells resulted in cytokine concentrations below detection limit. Data are presented as mean ± SD (*$p < 0.05$).

TLR2 and Nod2 synergize for the induction of cytokine release

In order to study the cross talk between TLR2 and Nod2 in the recognition of *M. tuberculosis*, we investigated whether mycobacterial TLR2 and Nod2 ligands synergize for the induction of cytokine release. Indeed, the Nod2 ligand MDP, the minimal component of peptidoglycan responsible for Nod2 activation, had strong synergistic effect on TNF production induced by the 19-kDa lipoprotein of *M. tuberculosis*, a specific mycobacterial TLR2 ligand (Figure 6A). Similar synergistic effects between the 19-kDa lipoprotein and MDP were observed when IL-6 (4.5-fold synergism), and IL-1β (7.2-fold synergism) were measured. This synergism was lost in individuals homozygous for the Nod2 3020insC mutation (Figure 6B) or macrophages harvested from TLR2−/− mice (5.7-fold synergism between lipoprotein 19-kDa and FK-156 in control macrophages, and 1.9-fold synergism in TLR2−/− cells). No such synergy was observed between MDP and mannosyl-caped lipoarabinomannan, a component of *M. tuberculosis* that does not interact with TLR2 (unpublished data).

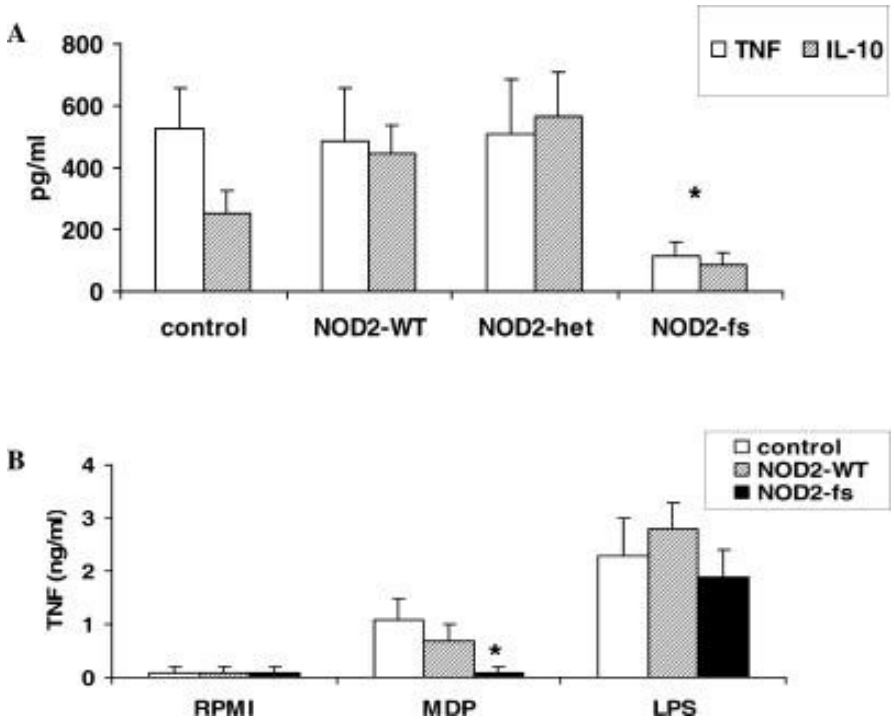

Figure 5. Human Nod2 is a receptor for *M. tuberculosis*. (A) MNCs isolated from four patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs), five patients heterozygous for Nod2 mutations (Nod2het), five patients with the wild-type Nod2 allele (Nod2wt), and five healthy volunteers with wild-type Nod2 (control) were stimulated with 10 µg/ml sonicated *M. tuberculosis*. TNF (white bars) and IL-10 (black bars) were measured after 24 h of stimulation at 37 °C by specific RIA and ELISA, respectively. (B) MNCs isolated from four patients with Crohn's disease homozygous for the 3020insC Nod2 mutation (Nod2fs, black bars), five patients with the wild-type Nod2 allele (Nod2wt, gray bars), and five healthy volunteers with wild-type Nod2 (control), were stimulated with 1 µg/ml MDP or 10 ng/ml LPS. TNF were measured after 24 h of stimulation at 37 °C by specific RIA. Medium-stimulated cells resulted in cytokine concentrations below detection limit. Data are presented as mean ± SD (*$p < 0.05$).

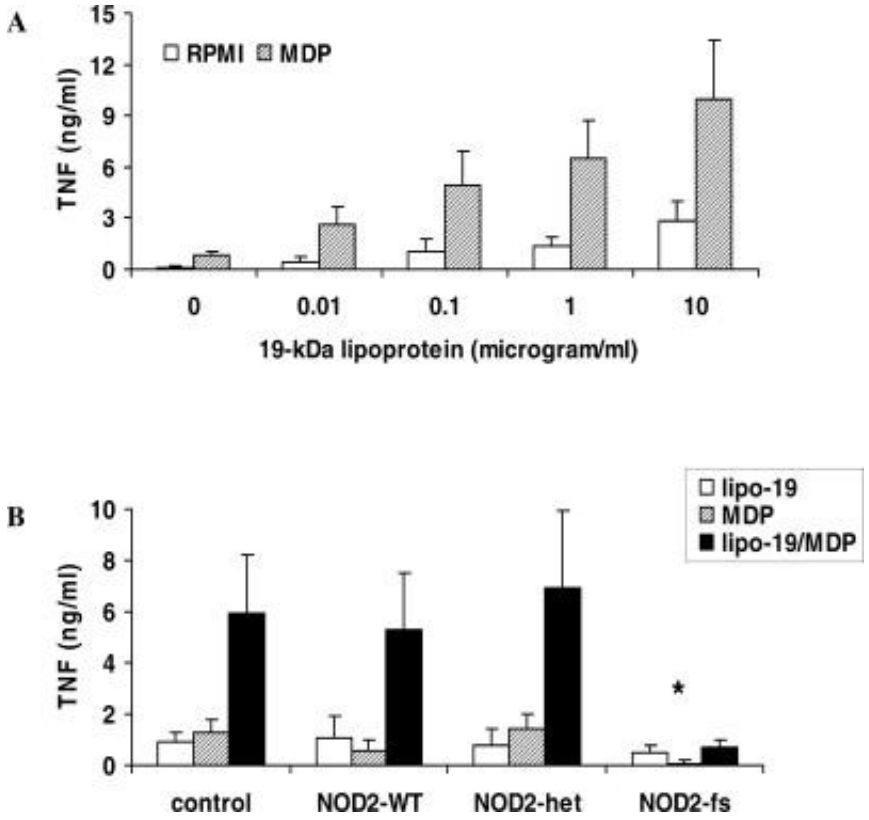

Figure 6. Nod2 and TLR2 have synergistic effects on cytokine production. (A) MNCs isolated from five healthy volunteers with wild-type Nod2 were co-stimulated with 1 µg/ml MDP and increasing concentrations of 19-kDa lipoprotein (indicated on the x-axis). TNF was measured after 24 h of stimulation at 37 °C by specific RIA. (B) The synergistic effects observed in the five control volunteers, as well as in five Crohn's disease patients heterozygous for Nod2 mutations (Nod2-het), and in five patients with the wild-type Nod2 allele (Nod2-WT), was lost in patients homozygous for the Nod2 3020insC mutation. Medium-stimulated cells resulted in cytokine concentrations below detection limit. Data are presented as mean ± SD (*$p < 0.05$).

Discussion

In the present study, we investigated the role of TLRs and NODs, the two most important classes of PRRs in the recognition by macrophages of *M. tuberculosis*. Although we confirmed the role of TLR2 and TLR4 for in mycobacterial recognition, strong residual activity was detectable in cells lacking TLR2, which suggests the existence of TLR-independent recognition mechanisms; this idea is supported by a study demonstrating MyD88-independent pathways of macrophage stimulation by *M. tuberculosis* [23]. Using cell lines transfected with Nod1 and Nod2, as well as primary MNCs defective in these receptors, we demonstrated that, in addition to TLRs, Nod2 represents a nonredundant recognition system of *M. tuberculosis*. We also demonstrated that mycobacterial TLR2 and Nod2 ligands synergize for the production of proinflammatory cytokines, and that this synergism is lost in cells lacking either of these receptors.

Several studies have demonstrated the role of TLR2 and TLR4 in the recognition of *M. tuberculosis*. The 19-kDa lipoprotein [24], lipomannan [25], and phosphatidyl-myo-inositol mannoside [26], all components of mycobacteria, have been identified as being responsible for TLR2-dependent activation, whereas heat-labile components associated with the cell wall were found to signal via TLR4 [27]. A role for TLRs in antimycobacterial defense was also suggested by the enhanced susceptibility to *M. tuberculosis* infection in mice deficient for MyD88, an adapter molecule shared by almost all TLR family members [28]. Similarly, TLR2−/− mice had a decreased clearance of the bacteria and developed chronic pneumonia when infected with low doses of microorganisms [15,29,30], whereas TLR4−/− mice showed variable responses to challenge with *M. tuberculosis* [31,32].

Our data confirm the important role played by TLRs, and especially TLR2, in the recognition of *M. tuberculosis*, but at the same time demonstrate strong TLR-independent induction of cytokines by this microorganism. The contribution of TLR4 was found to be less crucial in our study, and could be observed only when cells were stimulated with intact microorganisms.

Because *M. tuberculosis* is an intracellular pathogen, we hypothesized that intracellular recognition systems could contribute to the sensing of mycobacteria and stimulation of innate immunity. To test this hypothesis, we studied the effect of blocking the internalization of *M. tuberculosis* with cytochalasin B. Blocking internalization of *M. tuberculosis* partially inhibited *M. tuberculosis*-induced cytokine release in MNCs, whereas cytochalasin B potentiated the cytokine induction by zymosan, likely due to prolonged stimulation of receptors at the cell surface by zymosan. The differential effects of cytochalasin B on *M. tuberculosis*- or zymosan-induced cytokine secretion demonstrate that, in addition to the interaction with cell-membrane bound TLRs, *M. tuberculosis* is recognized by and induces cytokine production through intracellular receptors.

Nod2 and Nod1 are members of the expanding CATERPILLER family of proteins, which share an LRR domain similar to that found in TLRs [33]. Nod2 has been linked genetically to increased risk for Crohn's disease [34,35], and is a sensor of bacterial peptidoglycans [36]. When HEKs transfected with either Nod1 or Nod2 were stimulated with *M. tuberculosis* cell wall preparations, both of them—but most markedly those transfected with Nod2—showed a dose-dependent response. These data are consistent with the conclusion that Nod2 is a general sensor of bacteria through the detection of MDP, a peptidoglycan substructure present in bacterial cell walls [37]. However, the fact that Nod1 was found to be a poor sensor of *M. tuberculosis* not only in Nod1-transfected HEK cells, but also through the lack of a defective response in Nod1−/− macrophages, is somewhat puzzling. Indeed, Nod1 detects diaminopimelic acid (DAP)-type peptidoglycans, and earlier reports had suggested that *M. tuberculosis* peptidoglycan is of this category. Further investigation is therefore required to discover why Nod1 detects *M. tuberculosis* poorly.

To test whether the data in transfected cell lines could be reproduced in primary cells, we stimulated MNCs isolated from Crohn's disease patients homozygous for the 3020insC null-mutant allele with *M. tuberculosis*. This mutation leads to the deletion of the last 32 amino acids of the LRR region responsible for the detection of peptidoglycan, and we have recently shown that cells isolated from these patients are completely unable to recognize MDP or gram-positive peptidoglycan [38]. In line with the hypothesis that Nod2 is involved in the recognition of mycobacteria, both peritoneal macrophages from Nod2-deficient mice and MNCs isolated from patients homozygous for the 3020insC mutation of Nod2 were found to synthesize significantly less cytokines after stimulation with *M. tuberculosis*. Interestingly, the very strong defect in the response to *M. tuberculosis* of the cells of patients with the Nod2 3020insC mutation suggests that Nod1 is not able to compensate for the defective Nod2 recognition. This is in line with the weak stimulation of Nod1-transfected HEKs by *M. tuberculosis* and our recent finding that Nod2 is needed for normal signaling by Nod1 ligands such as Mur-Tri-DAP [39]. The reverse is not true in the case of Nod1: Macrophages harvested from Nod1−/− mice responded normally to *M. tuberculosis*, demonstrating that the absence of Nod1 can be compensated by other recognition systems, most likely Nod2.

The finding of strongly reduced cytokine production after stimulation with *M. tuberculosis* in cells of patients with a defective Nod2 and of Nod2 knock-out mice demonstrates that Nod2 is a key sensor of *M. tuberculosis* in mammalian cells. Interestingly, inhibition of both Nod2 and TLR2 systems blocked stimulation of cytokines by *M. tuberculosis* by substantially more than 50%. This suggests that the signaling pathways induced by these receptors interact and potentiate each other, and, conversely, that defects in one pathway lead to a loss of synergy. We have recently shown that Nod2 signals strongly synergize with specific TLR pathways such as TLR2, TLR4, and TLR3 [40]. We therefore investigated whether Nod2 activation leads to similar synergistic cytokine stimulation by TLR2 ligands specifically derived from *M. tuberculosis*. Indeed, MDP had strong synergistic effect on the cytokine production induced by the 19-kDa lipoprotein of *M. tuberculosis*, and this synergy was lost both in individuals

homozygous for the Nod2 3020insC mutation and in macrophages harvested from TLR2−/− mice.

An issue yet to be resolved is represented by the mechanism through which mycobacterial peptidoglycans come in contact with Nod2. Nod2 is an intracytoplasmic molecule, while *M. tuberculosis* remains located mainly in phagosomes. Although shedding of cell wall components from the microorganism is likely responsible for the release of peptidoglycans that are ultimately recognized by Nod2, the precise mechanism through which peptidoglycans translocate from the phagosome into the cytoplasm remains to be identified.

The data presented in this study demonstrate that Nod2 and TLRs are two nonredundant recognition mechanisms of *M. tuberculosis*. Both are essential for effective activation of the proinflammatory cytokine production by *M. tuberculosis*, and they strengthen each other's activity through synergistic effects. This demonstrates that host cells sense the presence of *M. tuberculosis* using multiple recognition systems in which different classes of receptors, in this case Nod2 and TLRs, interact with each other.

The involvement of Nod2 in the recognition of *M. tuberculosis* has several implications. First, it is possible that Nod2 is involved in recognition of other gram-positive bacteria with cell walls rich in peptidoglycans. The recent report of Nod2 serving as a receptor for *Streptococcus pneumoniae* supports this idea [41]. Second, our data suggest that Nod2 is involved in the recognition of other *Mycobacteria* species. From this point of view, *M. paratuberculosis* is of particular interest, due to its possible involvement in the pathogenesis of Crohn's disease [42]. A defective host defense against *M. paratuberculosis* in individuals bearing loss-of-function Nod2 mutations may be responsible for the invasion of the intestine and may promote chronic inflammation ultimately leading to Crohn's disease. This hypothesis is supported by the recently described presence of *M. paratuberculosis* in the circulation of patients with Crohn's disease [43]. Regarding the role of Nod2 in tuberculosis, one must, however, acknowledge that the present study provides information only on the in vitro recognition of *M. tuberculosis*, and studies investigating the role of Nod2 in infection models are warranted.

In conclusion, Nod2 and TLRs appear to serve as independent, nonredundant PRRs of *M. tuberculosis*. The intracellular pathways induced by Nod2 and TLR2 during recognition of mycobacterial components synergize, and the stimulation of cytokine production by *M. tuberculosis* is greatly impaired in individuals with Nod2 mutations.

Materials and methods

Reagents and microorganisms

Synthetic Pam3Cys and 19-kDa lipoprotein were purchased from EMC Microcollections (Tübingen, Germany). MDP and LPS (E. coli serotype 055:B5) were purchased from Calbiochem (San Diego, California, United States) and Sigma (St. Louis, Missouri, United States), respectively. The synthetic MDP was tested for contamination with lipoproteins or LPS in mice deficient in TLR2 or TLR4, respectively. No defect in cytokine production was apparent in these mice after stimulation with MDP, demonstrating the absence of contamination.

Cultures of *M. tuberculosis* H37Rv were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton-Dickinson, Palo Alto, California, United States), washed three times in sterile saline, and resuspended in RPMI 1640 medium at the various concentrations. Separate culture suspensions were sonicated for 10 min on ice, in order to obtain cell lysates.

Genotyping of Nod2 variants

Blood was collected from 74 patients with Crohn's disease and ten healthy volunteers. PCR amplification of Nod2 gene fragments containing the polymorphic site 3020insC was performed in 50-µl reaction volumes containing 100–200 ng of genomic DNA as previously described [44]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Four patients with Crohn's disease were found to be homozygous for the 3020insC mutation, and they were further investigated in the cytokine studies. As control groups, five patients with Crohn's disease who were heterozygous for the 3020insC Nod2 mutation, five patients with Crohn's disease bearing the wild-type allele, and five healthy volunteers homozygous for the wild-type Nod2 allele were included. None of the patients with Crohn's disease used immunosuppressive medication at the time of the study.

Isolation of MNCs and stimulation of cytokine production

After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10-ml EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). Isolation of MNCs was performed as described elsewhere [45], with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10 µg/ml gentamicin, 10 mM L-glutamine, and 10 mM pyruvate. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number was adjusted to 5×10^6 cells/ml.

MNCs (5×10^5) were added in a 100-µl volume to round-bottom 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) and incubated with either 100 µl of culture medium (negative control), or the various stimuli: M. tuberculosis bacteria (1×10^5 to 1×10^7 microorganisms/ml), *M. tuberculosis* sonicate (10 µg/ml), MDP (1 µg/ml), LPS (10 ng/ml), 19-kDa lipoprotein (concentrations as described below), or combinations of MDP and 19-kDa lipoprotein. The influence of internalization of *M. tuberculosis* on cytokine production was investigated by adding 20 µg/ml cytochalasin B during stimulation with the microorganism. Positive control stimulation for the effects of cytochalasin B was provided by stimulation of cells with zymosan (1 µg/ml; Sigma). All stimuli were checked for the contamination with LPS in the Limulus amoebocyte lysate assay and found to be negative. Cytochalasin B did not influence cell viability (unpublished data). To evaluate the role of TLR4 in the induction of cytokines, cells were preincubated with 10 µg/ml of a blocking monoclonal anti-TLR4 antibody (eBioscience, San Diego, California, United States).

Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from either ScCr (TLR4-defective) or C57Bl/10J (TLR4 control) mice, TLR2−/− or control TLR2+/+ mice (kindly provided by S. Akira, Osaka, Japan) [46], Nod1−/− and Nod1+/+ littermates (kindly provided by E. Abraham, Denver, Colorado, United States), or Nod2+/+ and Nod2−/− (from M. Giovannini, CEPH, Paris), backcrossed to the seventh generation into the C57BL6/J background, were harvested by injection of 4 ml of sterile PBS containing 0.38% sodium citrate [47]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 µg/ml gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner) at 1×10^5 cells/well, in a volume of 100 µl. The cells were stimulated with purified LPS (1 µg/ml), Pam3Cys (1 µg/ml), FK-156 ligand of murine Nod1 (1 µg/ml) as a control stimulation, or a sonicate of *M. tuberculosis* (1 µg/ml). After 24 h incubation at 37 °C, the supernatants were collected and stored at −70 °C until cytokine assays were performed.

Cytokine measurements

Human and murine TNFα concentrations were determined by specific RIAs as described [48,49]. IL-10 and IL-6 were measured by a commercial ELISA kits (Pelikine Compact, CLB, Amsterdam, The Netherlands), according to the instructions of the manufacturer.

Signaling through human TLR2 and TLR4 in transfected cell lines

CHOs stably transfected with human CD14 (3E10-CD14), a combination of CD14 and TLR2 (3E10-TLR2), or TLR4 (3E10-TLR4), were a kind gift from R. Ingalls [50]. All cell lines express inducible membrane CD25 under control of a region from the human E-selectin (ELAM-1) promoter containing NF-κB binding sites. Cells were maintained at 37 °C and 5% CO2 in HAM's F12 medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% FCS, 0.01% L-glutamine, 50 µg/ml gentamicin, and either 400 U/ml hygromycin and 0.5 mg/ml of G418 (for 3E10-TLR2) or 0.05 mg/ml of puromycin (for 3E10-TLR4) as additional selection antibiotics. TLR2 and TLR4 expression was confirmed by flow cytometry (Coulter

Epics XL-MCL, Beckman Coulter, Mijdrecht, the Netherlands) using PE-labeled anti-TLR2 (clone TL2.1) or anti-TLR4 (clone HTA125) (Immunosource, Halle-Zoersel, Belgium).

For stimulation experiments, 500 µl of cells in culture medium at a density of 1×10^5/ml were plated in 24-well culture plates. After an overnight incubation, cells were incubated with control medium, *M. tuberculosis* sonicate (10 µg/ml), Pam3Cys (10 µg/ml), or LPS (1 µg/ml) for 20 h, and thereafter cells were harvested using trypsin/EDTA (Cambrex, East Rutherford, New York, United States) and prepared for flow cytometry (Coulter FACS-scan). CD25 expression of the CHOs was measured using FITC-labeled anti-CD25 (DAKO, Glostrup, Denmark), and expressed as folds-over-mean increase.

Stimulation of Nod-transfected cell lines and NF-κB translocation

Studies examining the activation of NF-κB by *M. tuberculosis* in cells overexpressing Nod1 or Nod2 were carried out as previously described [51]. Briefly, 1×10^6/ml HEK293T cells were transfected overnight with 1 ng of either Nod1 or Nod2 plus 75 ng luciferase reporter plasmid. At the same time, heat-killed whole *M. tuberculosis* preparations (ratio of microorganisms and effector cells 1:10, 1:1, and 10:1) were added to cell culture medium, and the NF-κB–dependent luciferase activation was then measured following 24 h of incubation. NF-κB–dependent luciferase assays were performed in duplicate, and data represent three independent experiments[52].

Statistical analysis

The human experiments were performed in triplicate with blood obtained from patients and volunteers. The mouse experiments were performed twice in 10 mice per group, and the data are presented as cumulative results of all experiments performed. The differences between groups were analyzed by unpaired Student t-test, and where appropriate by paired t-test. The level of significance between groups was set at $p < 0.05$. The data are given as means ± standard deviation (SD).

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Reference List

1 Frieden,T.R. *et al.* (2003) Tuberculosis. *Lancet* 362, 887-899

2 van,C.R. *et al.* (2002) Innate immunity to Mycobacterium tuberculosis. *Clin. Microbiol. Rev.* 15, 294-309

3 Means,T.K. *et al.* (1999) Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J. Immunol.* 163, 3920-3927

4 Aliprantis,A.O. *et al.* (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* 285, 736-739

5 Vignal,C. *et al.* (2003) Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNFalpha and IL-8 secretion by a CD14-toll-like receptor 2-dependent mechanism. *J. Immunol.* 171, 2014-2023

6 Gilleron,M. *et al.* (2003) Acylation state of the phosphatidylinositol hexamannosides from Mycobacterium bovis bacillus Calmette Guerin and mycobacterium tuberculosis H37Rv and its implication in Toll-like receptor response. *J. Biol. Chem.* 278, 29880-29889

7 Nigou,J. *et al.* (2001) Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J. Immunol.* 166, 7477-7485

8 Geijtenbeek,T.B. *et al.* (2003) Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197, 7-17

9 Reiling,N. *et al.* (2002) Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol.* 169, 3480-3484

10 Kamath,A.B. *et al.* (2003) Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with Mycobacterium tuberculosis. *Infect. Immun.* 71, 4112-4118

11 Abel,B. *et al.* (2002) Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J. Immunol.* 169, 3155-3162

12 Branger,J. *et al.* (2004) Toll-like receptor 4 plays a protective role in pulmonary tuberculosis in mice. *Int. Immunol.* 16, 509-516

13 Drennan,M.B. *et al.* (2004) Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am. J. Pathol.* 164, 49-57

14 Reiling,N. *et al.* (2002) Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol.* 169, 3480-3484

15 Sugawara,I. *et al.* (2003) Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol. Immunol.* 47, 327-336

16 Fremond,C.M. *et al.* (2004) Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J. Clin. Invest* 114, 1790-1799

17 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

18 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

19 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

20 Girardin,S.E. *et al.* (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

21 Quesniaux,V. *et al.* (2004) Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes. Infect.* 6, 946-959

22 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

23 Shi,S. *et al.* (2003) MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to Mycobacterium tuberculosis. *J. Exp. Med.* 198, 987-997

24 Aliprantis,A.O. *et al.* (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* 285, 736-739

25 Vignal,C. *et al.* (2003) Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNFalpha and IL-8 secretion by a CD14-toll-like receptor 2-dependent mechanism. *J. Immunol.* 171, 2014-2023

26 Gilleron,M. *et al.* (2003) Acylation state of the phosphatidylinositol hexamannosides from Mycobacterium bovis bacillus Calmette Guerin and mycobacterium tuberculosis H37Rv and its implication in Toll-like receptor response. *J. Biol. Chem.* 278, 29880-29889

27 Means,T.K. *et al.* (1999) Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J. Immunol.* 163, 3920-3927

28 Fremond,C.M. *et al.* (2004) Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J. Clin. Invest* 114, 1790-1799

29 Reiling,N. *et al.* (2002) Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol.* 169, 3480-3484

30 Drennan,M.B. *et al.* (2004) Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am. J. Pathol.* 164, 49-57

31 Reiling,N. *et al.* (2002) Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol.* 169, 3480-3484

32 Kamath,A.B. *et al.* (2003) Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with Mycobacterium tuberculosis. *Infect. Immun.* 71, 4112-4118

33 Ting,J.P. and Davis,B.K. (2005) CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu. Rev. Immunol.* 23, 387-414

34 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

35 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

36 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

37 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

38 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

39 Netea,M.G. *et al.* (2005) The frameshift mutation in Nod2 results in unresponsiveness not only to Nod2- but also Nod1-activating peptidoglycan agonists. *J. Biol. Chem.* 280, 35859-35867

40 Netea,M.G. *et al.* (2005) Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J. Immunol.* 174, 6518-6523

41 Opitz,B. *et al.* (2004) Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J. Biol. Chem.* 279, 36426-36432

42 Greenstein,R.J. (2003) Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect. Dis.* 3, 507-514

43 Naser,S.A. *et al.* (2004) Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 364, 1039-1044

44 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

45 Endres,S. *et al.* (1988) Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin. Immunol. Immunopathol.* 49, 424-438

46 Takeuchi,O. *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11, 443-451

47 Kullberg,B.J. *et al.* (1993) Recombinant interferon-gamma enhances resistance to acute disseminated Candida albicans infection in mice. *J. Infect. Dis.* 168, 436-443

48 Drenth,J.P. *et al.* (1995) Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNFalpha and IL-1 beta production. *J. Appl. Physiol* 79, 1497-1503

49 Netea,M.G. *et al.* (1996) Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. *J. Clin. Invest* 97, 1366-1372

50 Lien,E. *et al.* (2000) Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest* 105, 497-504

51 Girardin,S.E. *et al.* (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

52 Girardin,S.E. *et al.* (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

Chapter 10

Mycobacterium paratuberculosis is recognized by Toll-like receptors and Nod2

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Abstract

Mycobacterium paratuberculosis has been suggested to be involved in the pathogenesis of Crohn's disease (CD). The importance of microorganisms in CD is supported by the association of CD with mutations in the intracellular pathogen recognition receptor (PRR) nucleotide-binding oligomerization domain 2 (Nod2). The aim of this study is to investigate the PRR involved in the recognition of *M. paratuberculosis*. Methods used include in vitro stimulation of transfected cell lines, murine macrophages, and human PBMC. *M. paratuberculosis* stimulated human TLR2 (hTLR2)-Chinese hamster ovary (CHO) cells predominantly and hTLR4-CHO cells modestly. Macrophages from TLR2 and TLR4 knockout mice produced less cytokines compared with controls after stimulation with *M. paratuberculosis*. TLR4 inhibition in human PBMC reduced cytokine production only after stimulation with live *M. paratuberculosis*. TLR-induced TNFα, IL-1ß and IL-10 production is mediated through MyD88, whereas Toll-IL-1R domain-containing adaptor inducing IFN-ß (TRIF) promoted the release of IL-1ß. hNod2-human embryo kidney (HEK) cells, but not hNod1-HEK cells, responded to stimulation with *M. paratuberculosis*. PBMC of individuals homozygous for the 3020insC Nod2 mutation showed a 70% defective cytokine response after stimulation with *M. paratuberculosis*. These results demonstrate that TLR2, TLR4, and Nod2 are involved in the recognition of *M. paratuberculosis* by the innate immune system.

Introduction

Mycobacteria are an important group of pathological microorganisms causing disease in humans. Almost 2 billion people are infected with *Mycobacterium tuberculosis* worldwide, and more than 2 million people die each year as a result of tuberculosis [1]. Other mycobacterial species, such as *Mycobacterium leprae*, are endemic in developing countries and are responsible for high morbidity and invalidate many people [2]. In developed countries, the prevalence of infections with mycobacteria is increasing, especially in patients with a compromised immune system. Under these circumstances, nonpathogenic mycobacteria may cause disease, such as *Mycobacterium avium* complex (MAC) in HIV-infected patients. *Mycobacterium paratuberculosis* is a member of the MAC, which has been suggested to be associated with Crohn's disease (CD), a chronic granulomatous inflammation of the gut. This association is controversial, but some findings support a causative role of *M. paratuberculosis* in the pathogenesis of CD [3]. In cows, *M. paratuberculosis* causes Johne's disease, which is very similar to CD in humans [4]. Although *M. paratuberculosis* is not a known pathogenic for humans, it can be cultured from intestinal biopsies of CD patients and even from the blood [5,6]. Furthermore, there is some evidence that treatment of CD with antibiotics has a beneficial effect on the disease activity [7,8].

It is thought that the innate immune response contributes to the difference in susceptibility to mycobacteria. The innate immune system constitutes the first line of defense against pathogens such as mycobacteria. The recognition of pathogen-associated molecular patterns by pathogen recognition receptors (PRR) is crucial for the initiation and coordination of this response. The importance of these receptors is emphasized further by the current concepts that the adaptive immune response is partly under control of the innate immune system [9,10]. The extracellular TLR [11,12] and C-type lectins [13,14] are membrane-bound PRR, which are complemented by the intracellular nucleotide-binding oligomerization domain-like receptor (NLR) family [15,16]. In animal models, it is well established that deficiencies of most of these receptors lead to an impaired immune response against pathogens and a higher susceptibility to infection [17].

Mutations in the LRR region of the nucleotide-binding oligomerization domain 2 (Nod2) gene, a member of the NLR family, are strongly associated with CD [18,19]. Nod2 is expressed in leukocytes and epithelial cells of the gastrointestinal tract [20], and this intracellular receptor can recognize peptidoglycans (PGNs) of Gram-positive and Gram-negative bacteria [21]. Stimulation of Nod2 can induce production of cytokines [22], chemokines [23], and defensins [24,25]. All these features suggest a key role for Nod2 in the innate immune response of the gastrointestinal tract. Not only does the intracellular PRR Nod2 seem to be important, but also, recognition of the commensal microflora and pathogens by extracellular TLRs in the gut is essential for the maintenance of the intestinal homeostasis and involved in the development of colitis [26]. Mutations in other genes of innate immunity have also been reported to be associated with susceptibility to CD [27,28], which is considered to have a

multifactorial pathogenesis, and there is consensus that the interaction of the innate immune system with the luminal bacterial flora is essential for developing the chronic inflammation of the intestinal mucosa [29]. However, it is still not clear whether pathogens, such as *M. paratuberculosis*, may cause or perpetuate inflammation in CD.

Despite the possible role of *M. paratuberculosis* in CD, there is not much known about the interaction of *M. paratuberculosis* and the innate immune system. A recent study has reported association of live M. paratuberculosis in blood cultures and biopsies with CD [6], and it has also been reported that CD patients with mutations in the Nod2 gene are infected more frequently with *M. paratuberculosis* than CD patients without these mutations [30]. Recently, we have shown that TLRs and Nod2 are nonredundant PRR of *M. tuberculosis* [31]. In the present study, we further explore the receptors involved in the recognition of the intracellular pathogen *M. paratuberculosis*. By using animal and human in vitro models, we were able to show that TLR2, TLR4, and Nod2 are involved in the recognition of *M. paratuberculosis*.

Results

M. paratuberculosis recognition by TLR2 and TLR4

To investigate the recognition of *M. paratuberculosis* by TLR2 and TLR4, both known to recognize *M. tuberculosis*, CHO cells transfected with human TLRs were stimulated with sonicated *M. paratuberculosis*. Whereas stimulation of CHO-TLR2 with *M. paratuberculosis* resulted in a robust expression of the reporter molecule CD25 (47±6%) as a measure of NF-κB activation (Fig. 1A), almost no expression of CD25 was seen after stimulation of CHO-TLR4 (8±3%; Fig. 1B). To verify these results in a more complex system, TLR knockout mice were used. Indeed, peritoneal macrophages of TLR2–/– mice produced significantly less cytokines compared with control mice after stimulation with M. paratuberculosis (Fig. 2).

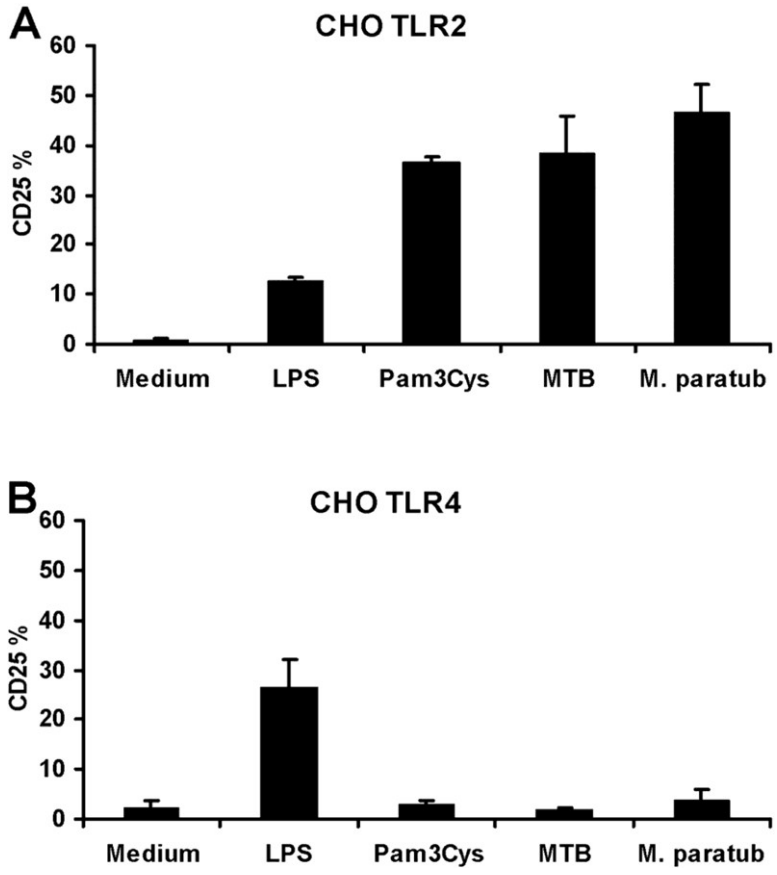

Figure 1. *M. paratuberculosis* stimulates TLR2-transfected CHO cells predominantly and TLR4-transfected CHO cells modestly, and CHO cells co-transfected with TLR2 and CD14 induced potent expression of CD25 on the cell membrane as a measure for NF-κB activation, after stimulation with a sonicated *M. tuberculosis* (MTB) or *M. paratuberculosis* (10 µg/ml) measured by FACS analysis (A). In contrast, cells transfected with CD14 and TLR4 were hardly activated by *M. tuberculosis* or *M. paratuberculosis* (B). LPS (1 µg/ml) and Pam3Cys (5 µg/ml) served as control stimuli for TLR4 and TLR2, respectively. Experiments were performed twice.

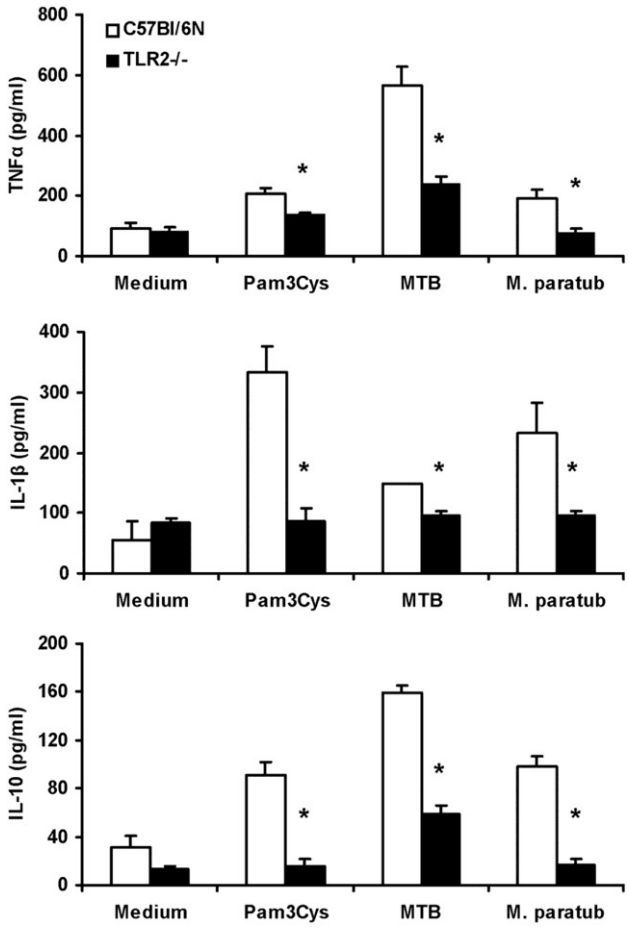

Figure 2. TLR2 mediates recognition of *M. paratuberculosis*. Peritoneal macrophages of TLR2–/– mice were stimulated with Pam3Cys (10 µg/ml), sonicated *M. tuberculosis* (10 µg/ml), or sonicated *M. paratuberculosis* (10 µg/ml). Cytokines TNFα, IL-1ß, and IL-10 were measured in the supernatant by ELISA or radioimmunoassay (RIA) after 24 h incubation at 37°C. Groups of five mice/group were stimulated. Data presented as mean ± SEM and were compared by Mann-Whitney U test (*, P<0.05).

After *M. paratuberculosis* stimulation, TLR4–/– murine macrophages also produced significantly less cytokines than controls (TNFα, Fig. 3A; IL-6, IL-10, and IL-1ß also reduced, not shown), although the difference was less pronounced, as seen in the macrophages of TLR2–/– mice. To further investigate the role of TLR4 in the recognition of *M. paratuberculosis*, human PBMC were stimulated with sonicated *M. paratuberculosis* in the presence and absence of anti-TLR4. Under these conditions, the TNFα production after stimulation with LPS was inhibited by over 50%, whereas no inhibitory effect on TNFα was seen when cells were stimulated with *M. paratuberculosis* (Fig. 3B). In addition, IL-1ß production induced by *M. paratuberculosis* was not influenced by TLR4 blockade (1255 pg/ml stimulation by *M. paratuberculosis* alone vs. 1860 pg/ml by *M. paratuberculosis* in the presence of the TLR4 antagonist). As heat-killing and sonication might influence the ability of *M. paratuberculosis* to stimulate TLR4, we next stimulated human PBMC with live *M. paratuberculosis*. Under these conditions, the cytokine production was decreased significantly in the presence of anti-TLR4 (Fig. 4). Thus, *M. paratuberculosis* is recognized by TLR2 and TLR4.

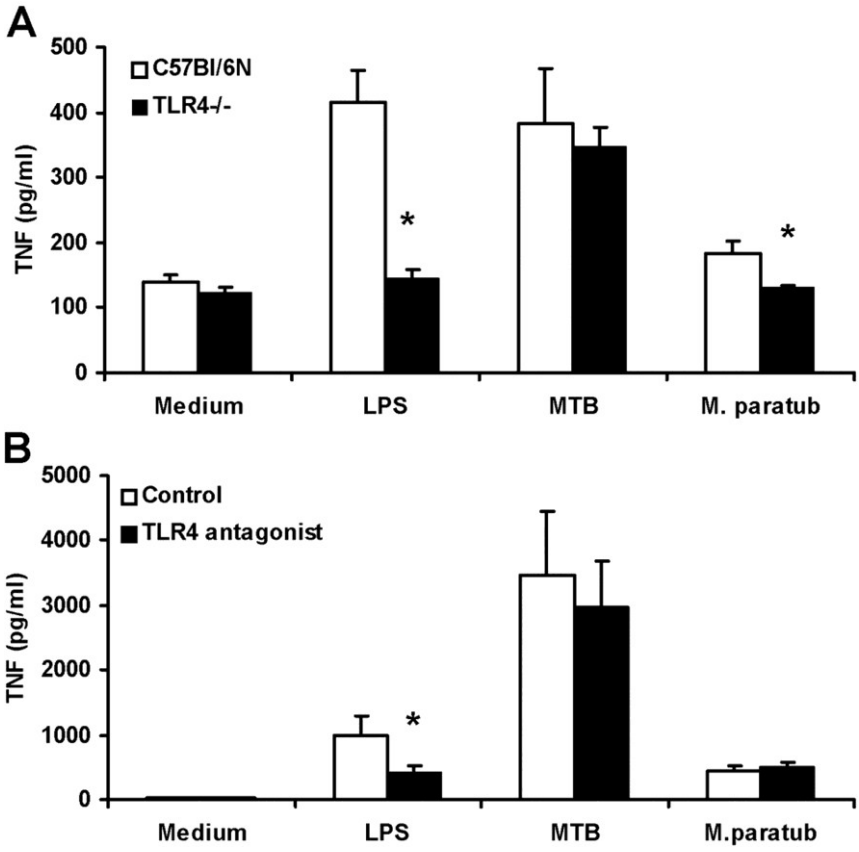

Figure 3. TLR4 involvement in *M. paratuberculosis* induced cytokine production. Peritoneal macrophages of TLR4–/– mice were stimulated with LPS (1 µg/ml), sonicated *M. tuberculosis* (10 µg/ml), or sonicated *M. paratuberculosis* (10 µg/ml). TNFα was measured in the supernatant by RIA after 24 h incubation at 37°C. Groups of five mice/group were stimulated (A). Human MNC of five healthy volunteers were stimulated with LPS (10 ng/ml), MTB (10 µg/ml), or sonicated *M. paratuberculosis* (10 µg/ml) in the presence or absence of TLR4 antagonist. TNFα was measured in the supernatant by ELISA after 24 h incubation at 37°C (B). Data presented as mean ± SEM and were compared by Mann-Whitney U test (mice) and Wilcoxon paired test (human; *, P<0.05).

As TLR2 and TLR4 are involved in the recognition of *M. paratuberculosis*, MyD88 and TRIF knockout mice were used to decipher which adaptor molecule is important for the cytokine signal pathway. Stimulation of peritoneal macrophages of MyD88–/– mice with *M. paratuberculosis* resulted in significant, reduced cytokine production compared with control (Fig. 5A). Peritoneal macrophages of TRIF–/– mice stimulated with *M. paratuberculosis* produced significantly less IL-1ß compared with control cells, whereas there was no difference in production of other cytokines (Fig. 5B). Thus, TLR signaling after stimulation with *M. paratuberculosis* is mediated through MyD88 and TRIF, although the MyD88-dependent pathway seems to be more important.

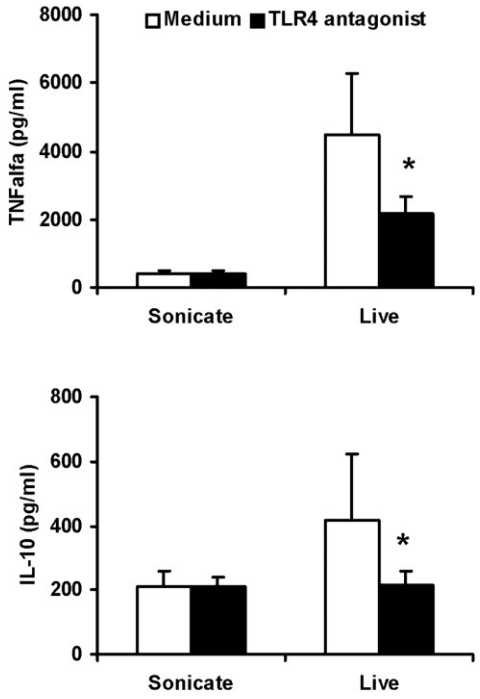

Figure 4. Live *M. paratuberculosis* stimulates TLR4 in human. Human MNC of five healthy volunteers were stimulated with sonicated *M. paratuberculosis* (10 µg/ml) or live *M. paratuberculosis* (10^7/ml) in the presence or absence of TLR4 antagonist. TNFα and IL-10 were measured in the supernatant by ELISA after 24 h incubation at 37°C. Data presented as mean ± SEM and compared by Wilcoxon paired test (*, P<0.05).

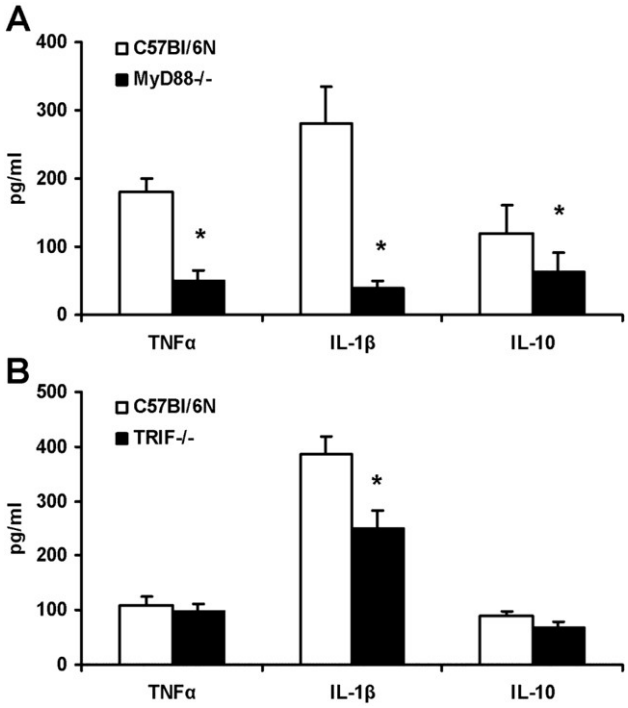

Figure 5. *M. paratuberculosis* induced cytokine production is TLR-adaptor molecules MyD88- and TRIF-dependent. Peritoneal macrophages of MyD88–/– mice (A) or TRIF–/– (B) were stimulated with sonicated *M. paratuberculosis* (10 µg/ml). Cytokines TNFα, IL-1ß, and IL-10 were measured in the supernatant by ELISA or RIA after 24 h incubation at 37°C. Groups of six mice/group were stimulated. Data presented as mean ± SEM and compared by Mann-Whitney U test (*, P<0.05).

M. paratuberculosis recognition of Nod1 and Nod2

To investigate the role of Nod1 and Nod2 in the recognition of *M. paratuberculosis*, HEK293 cells transfected with human Nod1 or Nod2 and a NF-κB-driven, luciferase-reporter system were stimulated with sonicated *M. paratuberculosis*. Nod2-transfected cells responded in a dose-dependent NF-κB activation, expressed as fold-increase luciferase activity, whereas Nod1-transfected cells responded marginally to high concentrations of *M. paratuberculosis* (Fig. 6). To explore the role of Nod2 recognition of *M. paratuberculosis* in human cells, MNC of CD patients homozygous for the 3020insC frameshift mutation (Nod2fs), known to be nonfunctional, were stimulated with sonicated *M. paratuberculosis* or a control TLR2 ligand (the lipopeptide Pam3Cys). When stimulated with Pam3Cys, TNFα production was similar in cells isolated from healthy volunteers (202±55 pg/ml), CD patients with wild-type Nod2 (213±35 pg/ml), and CD patients homozygous for the 3020insC mutation (175±53 pg/ml). After stimulation with *M. paratuberculosis*, cytokine production of Nod2fs cells was less than 30% of that by wild-type Nod2 allele (Nod2wt) and controls (Fig. 7). Thus, Nod2 is involved in the *M. paratuberculosis*-induced cytokine production by human primary PBMC.

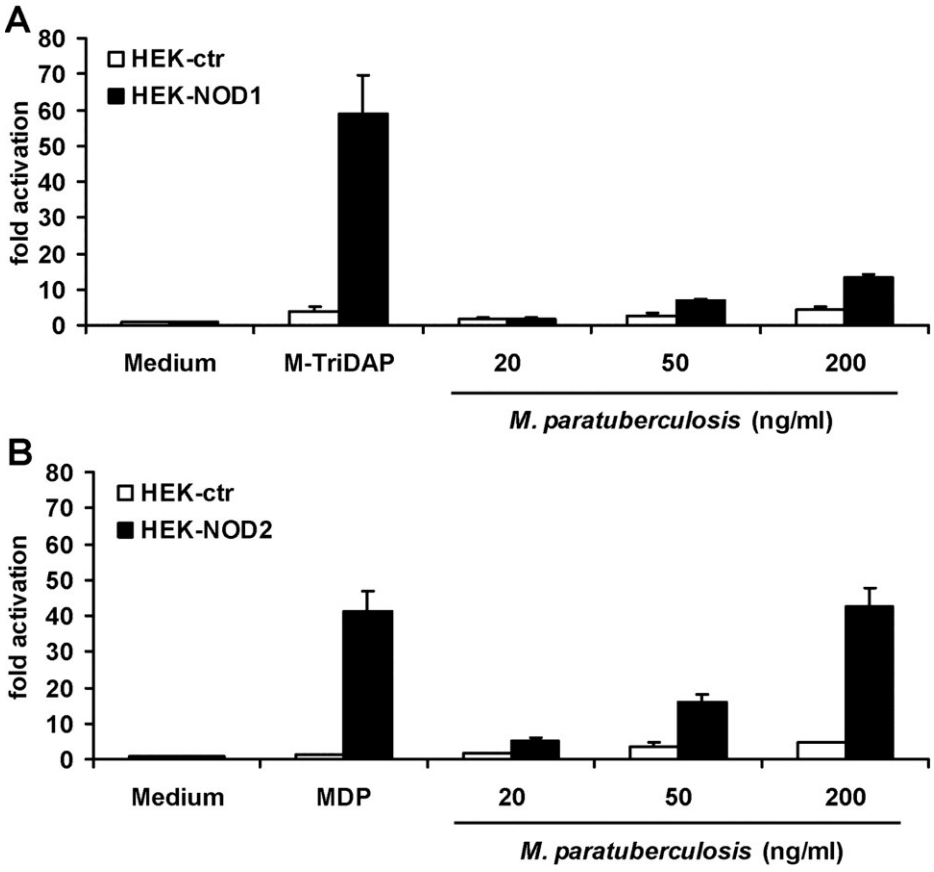

Figure 6. Nod1 and Nod2 recognize *M. paratuberculosis* in transfected HEK cells. HEK293 cells with a luciferase reporter construct (HEK-ctr) were transfected with Nod1 (A) or Nod2 (B) and stimulated with *M. paratuberculosis*. MDP (1 µg/ml) and TriDAP (1 µg/ml) served as control stimuli for Nod2 and Nod1, respectively. The NF-κB-dependent luciferase activation was measured following 24 h of incubation. NF-κB-dependent luciferase assays were performed in duplicate, and data represent three independent experiments.

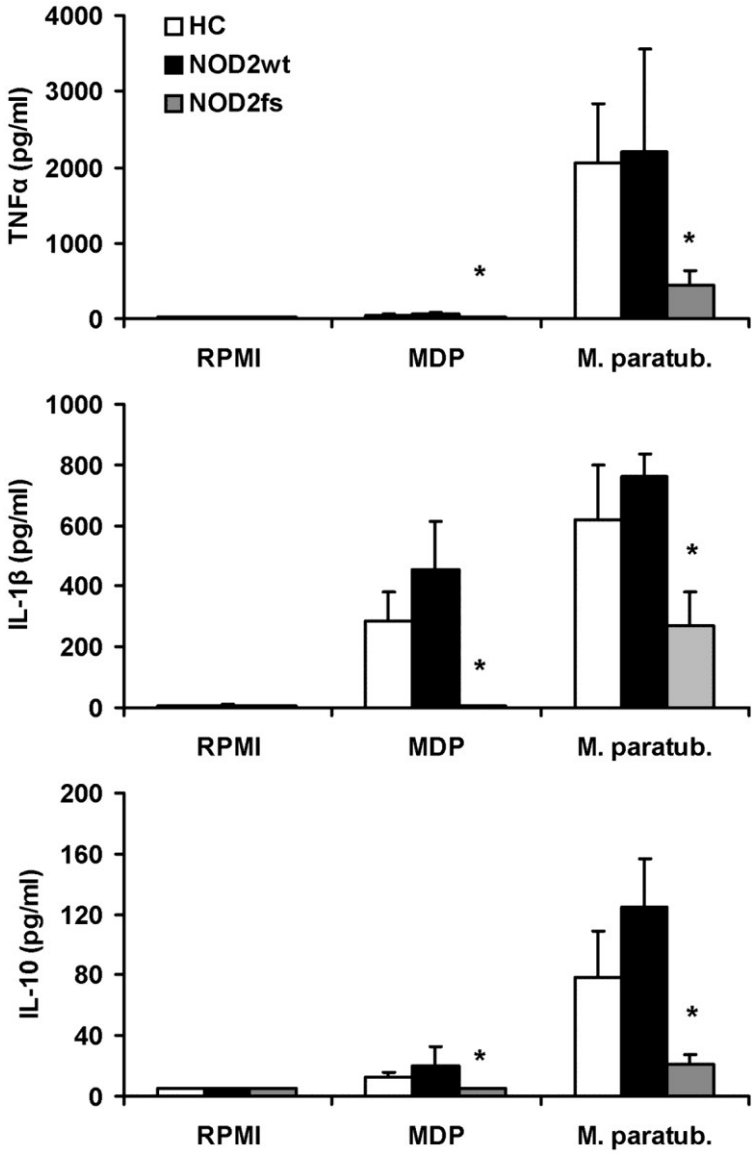

Figure 7. Human Nod2 is a receptor for *M. paratuberculosis*. MNC isolated from four patients with CD homozygous for the 3020insC Nod2 mutation (Nod2fs), five patients with the Nod2wt, and five healthy volunteers with wild-type Nod2 (HC) were stimulated with 10 µg/ml *M. paratuberculosis*. TNFα, IL-1ß, and IL-10 were measured after 24 h stimulation at 37°C by ELISA. MDP (100 nM), LPS (data not shown), and Pam3Cys (data not shown) served as control stimuli. Data are presented as means ± SEM and compared by Mann-Whitney U test (*, $P<0.05$).

Discussion

In this study, we demonstrate that TLR2, TLR4, and Nod2 are PRR for *M. paratuberculosis*, which are responsible for mediating cytokine production and stimulation of host defense. In the first part of the study, we show that murine and human TLR2 recognize sonicated *M. paratuberculosis*. The role of TLR2 for *M. tuberculosis* recognition was already established in different experimental models [31,32], and it is known that 19-kD lipoprotein of *M. tuberculosis* is recognized by TLR2 [33,34]. Which structure of *M. paratuberculosis* engages TLR2 is yet unknown, but various immune-active lipoproteins, such as19-kD lipoprotein [35], 22-kD lipoprotein [36], and 34-kD lipoprotein [37,38], have been identified in *M. paratuberculosis*. It is therefore tempting to speculate that *M. paratuberculosis* lipoproteins may be recognized by TLR2.

The role of TLR4 in the recognition of mycobacteria is more controversial. Sonicated *M. tuberculosis* has failed to stimulate human TLR4 in various experimental models. Conversely, it seems that TLR4 plays a beneficial role in tuberculosis infection models in mice [39,40], although not all groups could confirm these findings [41]. One remarkable aspect is that TLR4 responds differently to intact compared with sonicated mycobacteria [31], indicating that the sonication procedure changes the structure of certain cell wall components, which are important for TLR4 ligation. Our data show that TLR4 does not play a role in the recognition of sonicated *M. paratuberculosis* in human PBMC and transfected CHO cells. However, the role of TLR4 becomes apparent if human MNC are infected with live *M. paratuberculosis*, as under these conditions, cytokine responses were reduced by inhibition of TLR4. By contrast, macrophages from TLR4–/– mice did produce less TNFα after stimulation with sonicated *M. paratuberculosis*. This finding indicates that there are differences between the interaction of murine and human TLR4 with *M. paratuberculosis*. In addition, the interaction of leukocytes with components of live *M. paratuberculosis* at the level of the cell membrane is responsible for the TLR4-induced signaling. In contrast, Nod2 is an intracellular receptor, and recognition of *M. paratuberculosis* by Nod2 most likely requires prior internalization and digestion of the microorganism, leading to presentation of Nod2-stimulating motifs, as is probably the case in sonicated *M. paratuberculosis*. The structures of *M. paratuberculosis* responsible for interaction with human TLR4 are not known, but data obtained with *M. tuberculosis* indicate that structures sensitive for heat inactivation are responsible [32]. This might explain the different response after recognition of sonicated and living *M. paratuberculosis* by human TLR4.

The intracellular signals induced by TLRs leading to cytokine production are mediated by a limited number of adaptor molecules [42]. For TLR4, there are two intracellular pathways mediating its effects, represented by the adaptor molecules MyD88 and TRIF, whereas TLR2 depends entirely on the MyD88 pathway. We investigated which of the adaptor molecules are involved in the TLR signaling induced by *M. paratuberculosis*. The data from MyD88–/– and TRIF–/– mice showed that MyD88 is important for the induction of TNFα, IL-10, and IL-

1ß, whereas TRIF only seems to be important for the induction of IL-1ß. This is in line with early studies showing that TRIF is involved in the IL-1ß production [43]. In addition, TRIF-mediated signaling is associated mainly with IFN-ß production.

NLRs are a class of intracellular receptors, which complement the extracellular TLRs. Among the NLRs, Nod1 and Nod2 are the main receptors for PGN, and Nod1 recognizes the PGN of Gram-negative bacteria, whereas Nod2 recognizes the PGN of Gram-negative and Gram-positive bacteria. We have shown recently that Nod2 is involved in the recognition of *M. tuberculosis* and acts synergistically with TLR2 and TLR4 for the induction of cytokines [31]. When HEK cells transfected with Nod2 were stimulated with sonicated *M. paratuberculosis*, a dose-dependent effect was observed, suggesting that Nod2 recognizes *M. paratuberculosis*. In contrast, Nod1 only showed a marginal stimulation. We were able to confirm this finding in human primary cells. The MNC of CD patients homozygous for the 3020insC mutation, which are nonresponsive for MDP [44], had a significant, reduced cytokine response after stimulation with sonicated *M. paratuberculosis*. By sonicating *M. paratuberculosis* or during killing by macrophages, fragments of the complete bacteria will be exposed and be able to stimulate the different PRR. How small these components are and how far they are degraded still remain unclear. For stimulation of the intracellular receptors, these fragments have to be taken up by leukocytes and digested further by enzymes in the leukocytes before they can stimulate receptors such as Nod2. The PGN of mycobacteria contains muramyl peptides with the N-acetylglucosamine-N-glycolylmuramic acid backbone and the L-Ala-D-Glu-meso-DAP-D-Ala peptide side-chain [45]. This muramyl peptide contains motifs, such as L-Ala-D-Glu-meso-DAP (TriDAP), which can stimulate Nod1 [46]. Although it is known that the N-acetylmuramyl-L-Ala-D-Glu (MDP) structure is the minimal motif to stimulate Nod2 [46], our data also suggest that an N-glycocylmuramic acid-containing dipeptide can stimulate Nod2. As shown previously, the Nod2 3020insC mutation also leads to an impaired cytokine production after stimulation of Nod1 in human primary cells, suggesting that Nod1 and Nod2 are needed for an efficient response of the cells to Nod1 ligands [47]. Therefore, the lack of functional Nod2 in Crohn's patients with 3020insC mutation may lead to defective responses to mycobacterial PGNs, which contain strong Nod2-binding motifs but also weaker Nod1 ligands. In conclusion, as *M. paratuberculosis* is an intracellular pathogen, Nod2 might play an important role in the defense against this mycobacterium.

In the present study, we describe for the first time the receptors and mechanisms involved in the recognition of *M. paratuberculosis*. TLRs, such as TLR2 and TLR4, as well as Nod2 are independent recognition systems of *M. paratuberculosis*. It is tempting to speculate about the consequences of the finding that *M. paratuberculosis* is recognized by Nod2, as Nod2 polymorphism and *M. paratuberculosis* are associated with CD. One might hypothesize that a defective recognition of *M. paratuberculosis* by cells of Crohn's patients with Nod2 mutations is involved in the pathogenesis of CD.

Materials and methods

Reagents and microorganisms

Synthetic tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3Cys) was purchased from EMC Microcollections (Tübingen, Germany). Muramyl dipeptide (MDP) and LPS (Escherichia coli serotype 055:B5) were purchased from Calbiochem (San Diego, CA, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. The synthetic MDP was tested for contamination with lipoproteins or LPS in mice deficient in TLR2 or TLR4 (TLR2–/– or TLR4–/– respectively), demonstrating the absence of contamination. Diaminopimelic acid-containing muramyl tripeptide (M-TriDAP) was obtained as described previously [46]. As a specific TLR4 antagonist, we used LPS derived from the cell membrane of the Gram-negative bacterium *Bartonella quintana*, which has been demonstrated to be a highly specific TLR4 antagonist [48]. Cultures of *M. tuberculosis* H37Rv and *M. paratuberculosis* were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco Laboratories, Detroit, MI, USA), washed three times in sterile saline, and resuspended in RPMI-1640 medium at the various concentrations. Separate culture suspensions were washed three times in sterile saline; subsequently, the bacteria were heat-killed and disrupted in a bead-beater (Biospec Products, Bartlesville, OK, USA) using 0.1 mm glass beads.

Genotyping of Nod2 variants

Blood was collected from 154 patients with CD and 10 healthy volunteers. PCR amplification of Nod2 gene fragments containing the polymorphic site 3020insC was performed in 50 µl reaction volumes containing 100–200 ng genomic DNA as described previously [49]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 genetic analyzer according to the protocol of the manufacturer (Applied Biosystems, Nieuwerkerk, The Netherlands).

Seven patients with CD were found homozygous for the 3020insC frameshift mutation, and four of them were investigated further in the cytokine studies. As control groups, five patients with CD bearing the wild-type alleles and five healthy volunteers homozygous for the wild-type Nod2 allele were included.

Isolation of PBMC and stimulation of cytokine production

After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 ml EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). Isolation of mononuclear cells (MNC) was performed as described elsewhere [50] with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium RPMI 1640, supplemented with gentamicin 10 µg/ml, L-glutamine 10 mM, and pyruvate 10 mM. The

cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number was adjusted to 5 x 10^6 cells/ml.

MNC (5x10^5) in a 100-µl vol were added to round-bottom, 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) and incubated with 100 µl culture medium (negative control) or the various stimuli: *M. tuberculosis* sonicate (10 µg/ml), *M. paratuberculosis* sonicate (10 µg/ml), MDP (100 nM), LPS (10 ng/ml), or TLR4 antagonist (10 µg/ml).

Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from TLR4–/–, TLR2–/–, MyD88–/–, or control C57Bl/6N mice (kindly provided by Dr. Shizuo Akira, Research Institute for Microbiological Diseases, Osaka University, Osaka, Japan) [51] and LPS2 [Toll-IL-1R domain-containing adaptor-inducing IFN-ß (TRIF)-defective] mice (kindly provided by Dr. Bruce Beutler, The Scripps Institute, La Jolla, CA, USA) [52] were harvested by injection of 4 ml sterile PBS containing 0.38% sodium citrate [53]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 µg/ml gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn) at 1 x 10^5 cells/well in a volume of 100 µl. The cells were stimulated with purified LPS (1 µg/ml), Pam3Cys (1 µg/ml), sonicate of *M. tuberculosis* (10 µg/ml), or sonicate of *M. paratuberculosis* (10 µg/ml). After 24 h incubation at 37°C, the supernatants were collected and stored at –70°C until cytokine assays were performed.

Cytokine measurements

Murine TNFα and IL-1ß concentrations were determined by specific radioimmunoassays as described [54,55]. Murine IL-10 and human IL-10 and IL-1ß were measured by commercial ELISA kits (Pelikine Compact, Sanquin, Amsterdam, The Netherlands), according to the instructions of the manufacturer. Human TNFα concentrations were determined by specific ELISA [56].

Signaling through human TLR2 and TLR4 in transfected cell lines

Chinese hamster ovary (CHO) fibroblasts stably transfected with human CD14 (3E10-CD14), a combination of CD14 and TLR2 (3E10-TLR2) or TLR4 (3E10-TLR4), were a kind gift from Dr. Robin Ingalls (Boston Medical Center, Boston University School of Medicine, Boston, MA, USA). Cell lines express inducible membrane CD25 under control of a region from the human E-selectin (endothelial leukocyte-adhesion molecule 1) promoter containing NF-κB-binding sites. Cells were maintained at 37°C and 5% CO2 in HAM's F12 medium (Gibco, Invitrogen, Breda, the Netherlands), supplemented with 10% FCS, 0.01% L-glutamine, 50 µg/mL gentamicin and 400 U/mL hygromycin, and 0.5 mg/mL G418 (for 3E10-TLR2) or 0.05 mg/mL puromycine (for 3E10-TLR4) as additional selection antibiotics. TLR2 and TLR4 expression was confirmed by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter, Mijdrecht, the Netherlands) using PE-labeled anti-TLR2 (Clone TL2.1) or anti-TLR4 (Clone HTA125; Immunosource, Halle-Zoersel, Belgium).

For stimulation experiments, 500 µl cells in culture medium at a density of 1 x 10^5/mL were plated in 24-well culture plates. After an overnight incubation, cells were incubated with control medium, *M. tuberculosis* sonicate (10 µg/ml), *M. paratuberculosis* sonicate (10 µg/ml), Pam3Cys (10 µg/ml), or LPS (1 µg/ml) for 20 h, and thereafter, cells were harvested using trypsin/EDTA (Cambrex, East Rutherford, NY, USA) and prepared for flow cytometry (Coulter FACSscan). CD25 expression of the CHO cells was measured using FITC-labeled anti-CD25 (Dako, Glostrup, Denmark) and expressed as a percentage of CD25-positive cells.

Stimulation of NOD cell lines and NF-κB translocation

Studies examining the activation of NF-κB by M. paratuberculosis sonicates in cells overexpressing Nod1 or Nod2 were carried out as described previously [57]. Briefly, human embryo kidney (HEK)293T cells were transfected overnight with 1 ng Nod1 or Nod2 plus 75 ng luciferase reporter plasmid. At the same time, sonicated, heat-killed *M. paratuberculosis* preparations were added to cell culture medium, and the NF-κB-dependent luciferase activation was then measured following 24 h of incubation. NF-κB-dependent luciferase assays were performed in duplicate, and data represent three independent experiments and expressed as a folds-over-mean increase.

Statistical analysis

The human experiments were performed in triplicate with blood obtained from patients and volunteers. The mouse experiments were performed twice in 10 mice per group, and the data are presented as cumulative results of all experiments performed. The differences between groups were analyzed by Mann-Whitney U test or Wilcoxon and where appropriate, by Kruskal-Wallis ANOVA test. The level of significance between groups was set at $P < 0.05$. The data are given as means ± SEM.

Reference List

1 Frieden,T.R. *et al.* (2003) Tuberculosis. *Lancet* 362, 887-899

2 Engers,H. and Morel,C.M. (2003) Leprosy. *Nat. Rev. Microbiol.* 1, 94-95

3 Shanahan,F. and O'Mahony,J. (2005) The mycobacteria story in Crohn's disease. *Am. J. Gastroenterol.* 100, 1537-1538

4 Chacon,O. *et al.* (2004) Johne's disease, inflammatory bowel disease, and Mycobacterium paratuberculosis. *Annu. Rev. Microbiol.* 58, 329-363

5 Naser,S.A. *et al.* (2002) In situ identification of mycobacteria in Crohn's disease patient tissue using confocal scanning laser microscopy. *Mol. Cell Probes* 16, 41-48

6 Naser,S.A. *et al.* (2004) Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 364, 1039-1044

7 Hulten,K. *et al.* (2000) Antibacterial therapy for Crohn's disease: a review emphasizing therapy directed against mycobacteria. *Dig. Dis. Sci.* 45, 445-456

8 Shafran,I. *et al.* (2002) Open clinical trial of rifabutin and clarithromycin therapy in Crohn's disease. *Dig. Liver Dis.* 34, 22-28

9 Janeway,C.A., Jr. and Medzhitov,R. (1998) Introduction: the role of innate immunity in the adaptive immune response. *Semin. Immunol.* 10, 349-350

10 Medzhitov,R. and Janeway,C.A., Jr. (1998) Innate immune recognition and control of adaptive immune responses. *Semin. Immunol.* 10, 351-353

11 Takeda,K. *et al.* (2003) Toll-like receptors. *Annu. Rev. Immunol.* 21, 335-376

12 Medzhitov,R. *et al.* (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388, 394-397

13 Figdor,C.G. *et al.* (2002) C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol.* 2, 77-84

14 McGreal,E.P. *et al.* (2005) Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr. Opin. Immunol.* 17, 18-24

15 Ting,J.P. and Davis,B.K. (2005) CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu. Rev. Immunol.* 23, 387-414

16 Inohara,N. and Nunez,G. (2003) NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* 3, 371-382

17 Netea,M.G. *et al.* (2004) Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. *J. Leukoc. Biol.* 75, 749-755

18 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

19 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

20 Ogura,Y. *et al.* (2003) Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 52, 1591-1597

21 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

22 Netea,M.G. *et al.* (2005) Nucleotide-Binding Oligomerization Domain-2 Modulates Specific TLR Pathways for the Induction of Cytokine Release. *J. Immunol.* 174, 6518-6523

23 van Heel,D.A. *et al.* (2005) Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 365, 1794-1796

24 Voss,E. *et al.* (2006) NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. *J. Biol. Chem.* 281, 2005-2011

25 Wehkamp,J. *et al.* (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53, 1658-1664

26 Rakoff-Nahoum,S. *et al.* (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229-241

27 Duerr,R.H. *et al.* (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314, 1461-1463

28 Cantor,M.J. *et al.* (2005) The role of cytokine gene polymorphisms in determining disease susceptibility and phenotype in inflammatory bowel disease. *Am. J. Gastroenterol.* 100, 1134-1142

29 Podolsky,D.K. (2002) Inflammatory bowel disease. *N. Engl. J. Med.* 347, 417-429

30 Sechi,L.A. *et al.* (2005) Mycobacterium avium subsp. paratuberculosis, genetic susceptibility to Crohn's disease, and Sardinians: the way ahead. *J. Clin. Microbiol.* 43, 5275-5277

31 Ferwerda,G. *et al.* (2005) NOD2 and toll-like receptors are nonredundant recognition systems of Mycobacterium tuberculosis. *PLoS. Pathog.* 1, 279-285

32 Means,T.K. *et al.* (1999) Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J. Immunol.* 163, 3920-3927

33 Gehring,A.J. *et al.* (2003) The Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits gamma interferon-regulated HLA-DR and Fc gamma R1 on human macrophages through Toll-like receptor 2. *Infect. Immun.* 71, 4487-4497

34 Hertz,C.J. *et al.* (2001) Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J. Immunol.* 166, 2444-2450

35 Huntley,J.F. *et al.* (2005) Immunoreactivity of the Mycobacterium avium subsp. paratuberculosis 19-kDa lipoprotein. *BMC. Microbiol.* 5, 3

36 Dupont,C. *et al.* (2005) Identification and characterization of an immunogenic 22 kDa exported protein of Mycobacterium avium subspecies paratuberculosis. *J. Med. Microbiol.* 54, 1083-1092

37 Silbaq,F.S. *et al.* (1998) Characterization of a 34-kilodalton protein of Mycobacterium leprae that is isologous to the immunodominant 34-kilodalton antigen of Mycobacterium paratuberculosis. *Infect. Immun.* 66, 5576-5579

38 Gioffre,A. *et al.* (2006) Lpp34, a novel putative lipoprotein from Mycobacterium avium subsp. paratuberculosis. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 53, 34-41

39 Abel,B. *et al.* (2002) Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J. Immunol.* 169, 3155-3162

40 Heldwein,K.A. *et al.* (2003) TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. *J. Leukoc. Biol.* 74, 277-286

41 Shim,T.S. *et al.* (2003) Toll-like receptor 4 plays no role in susceptibility of mice to Mycobacterium tuberculosis infection. *Tuberculosis. (Edinb.)* 83, 367-371

42 Beutler,B. (2004) Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 430, 257-263

43 Toshchakov,V.U. *et al.* (2005) Differential involvement of BB loops of toll-IL-1 resistance (TIR) domain-containing adapter proteins in. *J. Immunol.* 175, 494-500

44 Netea,M.G. *et al.* (2005) NOD2 3020insC mutation and the pathogenesis of Crohn's disease: impaired IL-1beta production points to a loss-of-function phenotype. *Neth. J. Med.* 63, 305-308

45 Adam,A. *et al.* (1974) Adjuvant activity of monomeric bacterial cell wall peptidoglycans. *Biochem. Biophys. Res. Commun.* 56, 561-567

46 Girardin,S.E. *et al.* (2003) Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J. Biol. Chem.* 278, 41702-41708

47 Netea,M.G. *et al.* (2005) The frameshift mutation in Nod2 results in unresponsiveness not only to Nod2- but also Nod1-activating peptidoglycan agonists. *J. Biol. Chem.* 280, 35859-35867

48 Abdollahi-Roodsaz,S. *et al.* (2007) Inhibition of TLR4 Breaks the Inflammatory Loop in Autoimmune Destructive Arthritis. *Arthritis Rheum.*

49 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

50 Endres,S. *et al.* (1988) Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin. Immunol. Immunopathol.* 49, 424-438

51 Takeuchi,O. *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11, 443-451

52 Hoebe,K. *et al.* (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424, 743-748

53 Kullberg,B.J. *et al.* (1993) Recombinant interferon-gamma enhances resistance to acute disseminated Candida albicans infection in mice. *J. Infect. Dis.* 168, 436-443

54 Drenth,J.P. *et al.* (1995) Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNFalpha and IL-1 beta production. *J. Appl. Physiol* 79, 1497-1503

55 Netea,M.G. *et al.* (1996) Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. *J. Clin. Invest* 97, 1366-1372

56 Grebenchtchikov,N. *et al.* (2005) Development of a sensitive ELISA for the quantification of human tumour necrosis factor-alpha using 4 polyclonal antibodies. *Eur. Cytokine Netw.* 16, 215-222

57 Girardin,S.E. *et al.* (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

Chapter 11

Crohn's disease patients homozygous for the 3020insC Nod2 mutation have a defective Nod2/TLR4 cross-tolerance to intestinal stimuli

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Abstract

Mutations in nucleotide-binding oligomerization domain-2 (Nod2), leading to defective recognition of bacterial peptidoglycans, are associated with Crohn's disease. The underlying mechanism that results in increased inflammation in the guts of the patients bearing Nod2 mutations is still unclear. We hypothesized that Nod2 engagement leads to cross-tolerance to stimulation of Toll-like receptors (TLR), and we investigated whether patients with Crohn's disease who bear Nod2 mutations display a disturbed Nod2/TLR cross-tolerance. Peripheral blood mononuclear cells preincubated with Nod2 ligands were specifically down-regulated for the production of tumor necrosis factor-α (TNFα) induced by the TLR4 ligand lipopolysaccharide, as well as by intestinal microorganisms, whereas the production of anti-inflammatory cytokines was not modulated. While in cells isolated from patients with Crohn's disease with the wild-type Nod2 allele, the Nod2 engagement led to a similar cross-tolerance to TLR4-dependent stimulation of TNFα, the cross-tolerance between Nod2 and TLR4 was absent in the cells of five patients homozygous for the 3020insC Nod2 mutation, leading to uninhibited release of TNFα by TLR4 ligands and intestinal bacteria. In conclusion, we propose the absence of Nod2/TLR4 cross-tolerance as a central mechanism for the increased susceptibility to Crohn's disease in individuals with Nod2 mutations.

Introduction

The IBD1 locus on chromosome 16 has been identified as a susceptibility locus for Crohn's disease [1], and subsequently, nucleotide-binding oligomerization domain-2 (Nod2) has been identified as the candidate gene within the IBD1 locus [2-4]. Nod2 is a member of the NACHT-LRR receptor (NLR) protein family, which is known to be involved in recognition of microbial structures, and is expressed intracellularly in antigen-presenting cells [5]. Subsequent studies have identified Nod2 as the intracellular receptor for the muramyl dipeptide (MDP) component of peptidoglycan [6,7]. However, the pathophysiological mechanisms responsible for the increased susceptibility to Crohn's disease in patients with Nod2 mutations are unclear. Crohn's disease patients who are homozygous for the 3020insC mutation of the Nod2 gene were found to have a defective release of cytokines after stimulation with peptidoglycan and its MDP components [8,9]. How this defect paradoxically translates into increased local inflammation in the intestine has not been clarified. Several potential mechanisms have been proposed, including defective release of anti-inflammatory cytokines [8], or defective production of defensins, leading to bacterial overgrowth [10,11]. Whereas a gain-of-function effect of the mutation on interleukin-1β (IL-1β) release was demonstrated in a mouse model [12], the latter mechanism was refuted in human studies [13]. None of these mechanisms satisfactorily explains the increased inflammatory reaction found in the intestines of patients with Crohn's disease.

In addition to its role as receptor for peptidoglycan, Nod2 also modulates signaling induced by Toll-like receptors (TLRs). Several studies have suggested that concomitant stimulation of Nod2 and TLR synergistically induces cytokines, although controversy exists around this issue [9,14]. Such synergism between different receptors is a known amplification mechanism aimed at enforcing innate immunity. In addition, tolerance may be induced by repeated or chronic exposure to a stimulus. Such tolerance has been extensively described for bacterial lipopolysaccharide (LPS, endotoxin), and cross-tolerance to LPS and other stimuli at the level of TLR2 and TLR4 has been established [15,16]. It is not known whether stimulation of Nod2 is able to induce cross-tolerance to TLR signaling. If this were the case, the chronic stimulation of Nod2 by intestinal peptidoglycans could tolerize for subsequent bacterial stimulation of TLRs, resulting in down-modulation of the proinflammatory cytokine response. Such a mechanism may contribute to maintaining intestinal homeostasis in the presence of a large quantity of bacterial stimuli.

Here we describe five patients who are homozygous for the 3020insC Nod2 mutation, and we demonstrate that Nod2 engagement leads to cross-tolerance to TLR4-dependent production of tumor necrosis factor-α (TNFα), the central cytokine mediating inflammation in Crohn's disease. The cross-tolerance between Nod2 and TLR4 was absent in the patients with the Nod2 mutation, and this leads to uninhibited release of TNFα by intestinal bacteria and other TLR4 ligands. The tolerizing effect of Nod2 is specific for TLR4 stimulation; stimulation by TLR2 ligands was not affected.

Results

Nod2-induced cross-tolerance for TLR4-induced TNFα

Prestimulation of peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers with various concentrations of MDP significantly reduced their capacity to synthesize TNFα after stimulation of TLR4 by LPS, but not after stimulation with the TLR2 ligands Pam3Cys or MALP (Fig. 1a). To investigate whether MDP can also induce tolerance to intestinal pathogens, cells exposed to MDP were subsequently stimulated with heat-killed *S. typhimurium* or *B. fragilis*. Although both are intestinal microorganisms, *S. typhimurium* induces intracellular signals through both TLR4 and TLR2 [17], whereas TLR2 is the main receptor mediating the cytokine production induced by *B. fragilis* [18]. In line with the results obtained with purified TLR ligands, stimulation of Nod2 by MDP led to tolerance to the induction of TNFα by *S. typhimurium* (Fig. 1b). The effect of Nod2 engagement was less pronounced on the stimulation of cytokines by *B. fragilis* (Fig. 1b).

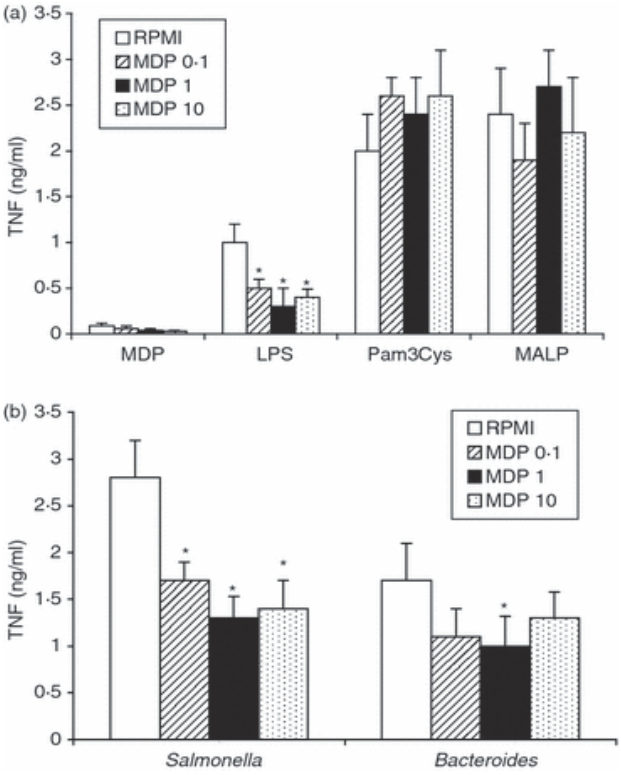

Figure 1. Nod2 induces cross-tolerance for TLR4-induced TNFα. (a) Mononuclear cells isolated from five healthy volunteers were preincubated for 24 hr with various concentrations of MDP (0.1, 1 and 10 μg/ml), and subsequently stimulated for 24 hr with LPS (1 ng/ml), Pam3Cys (10 μg/ml) or MALP (10 μg/ml). (b) To investigate whether MDP can also induce tolerance to stimulation with intestinal pathogens, mononuclear cells tolerized with MDP in various concentrations (see above) were subsequently stimulated with heat-killed *S. typhimurium* or *B. fragilis* (both at a concentration of 1×10^6 microorganisms/ml). Concentrations of TNFα were measured by enzyme-linked immunosorbent assay. Data are presented as mean ± SD, and compared by Wilcoxon paired test (n = 5, *P < 0.05).

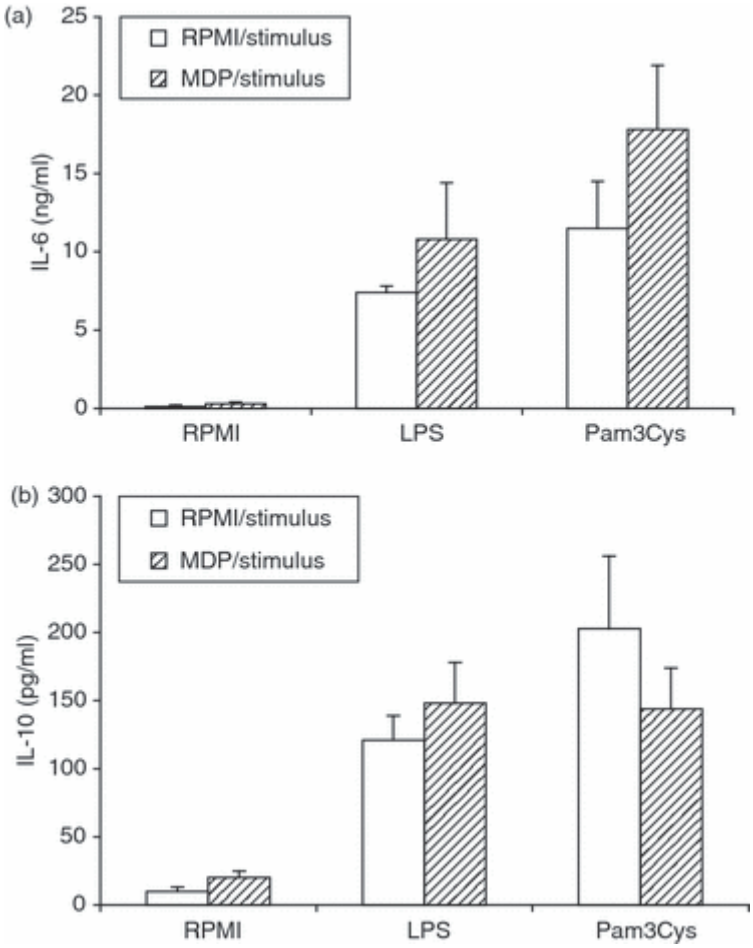

Figure 2. Nod2 does not influence the induction of IL-6 and IL-10 stimulated by TLRs. MNC isolated from five healthy volunteers were preincubated for 24 hr with MDP (10 µg/ml), and subsequently stimulated for another 24 hr with LPS (1 ng/ml) or Pam3Cys (10 µg/ml). Concentrations of IL-6 (a) and IL-10 (b) were measured by enzyme-linked immunosorbent assay. Data are presented as mean ± SD, and compared by Wilcoxon paired test (n = 5, all differences not significant).

In contrast to TNF production, the synthesis of IL-6 in response to the various stimuli was not changed by the preincubation of PBMC with MDP (Fig. 2). Similarly, IL-10 production induced by TLR2 or TLR4 ligands was not changed by preincubation of PBMC with MDP (Fig. 2). Production of IL-12 was undetectable in all the samples, irrespective of the stimulus used.

Presence of bacterial peptidoglycans in the intestinal wall

The hypothesis that Nod2 signals induce tolerance for subsequent stimulation of TLR would require the presence of sufficient amounts of peptidoglycan in enterocytes. Indeed, immunostaining for peptidoglycans clearly demonstrated the intracellular presence of peptidoglycan in colonic enterocytes, both in healthy individuals (Fig. 3a) and in patients with Crohn's disease (Fig. 3b).

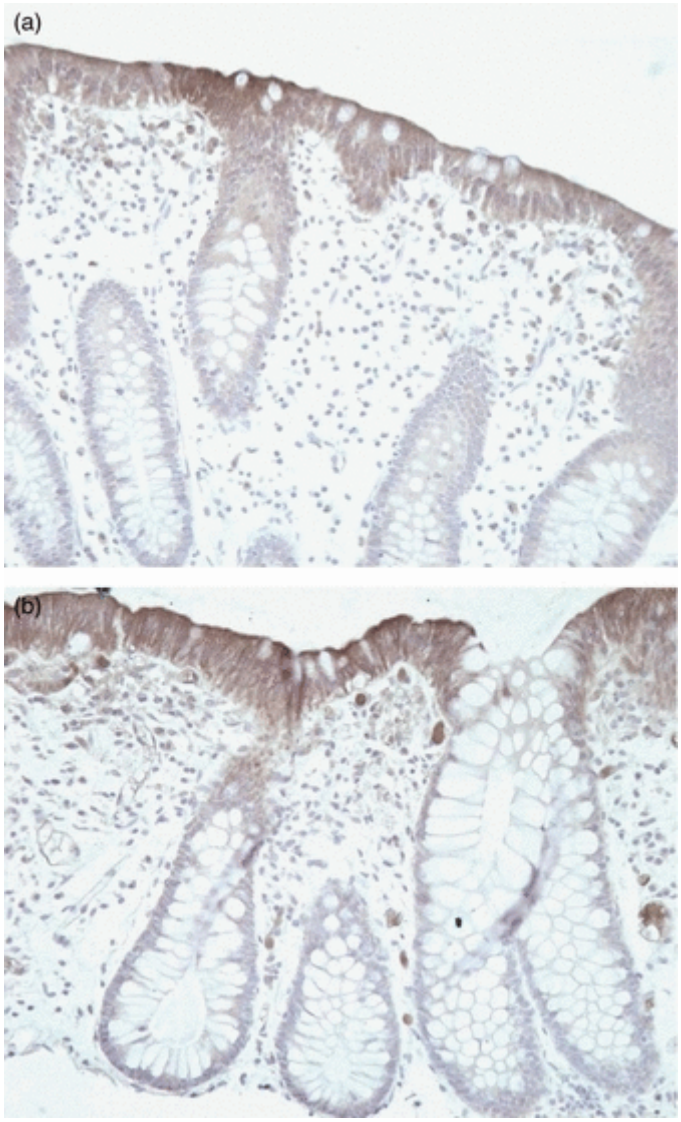

Figure 3. Peptidoglycan is present in the intestinal mucosa. Immunostaining using a monoclonal mouse anti-MDP antibody (0.3 µg/ml) was performed in the colonic mucosa of healthy individuals (a) and patients with Crohn's disease (b). The secondary antibody, biotinylated rabbit anti-mouse immunoglobulin G, was added to the incubation for 30 min. Slides were stained with streptavidin peroxidase, developed with DAB, and counterstained with haematoxylin for 30 seconds (magnification 400×).

Nod2/TLR4 cross-tolerance in Crohn's disease patients with a 3020insC Nod2 mutation

Nod2-induced cross-tolerance on TLR4-mediated TNFα production was investigated in volunteers, Crohn's disease patients with the wild-type Nod2 allele (Nod2wt), and patients who were homozygous for the 3020insC mutation (Nod2fs). Cross-tolerance between Nod2 and TLR4 was only absent in patients with the Nod2fs mutation (Fig. 4).

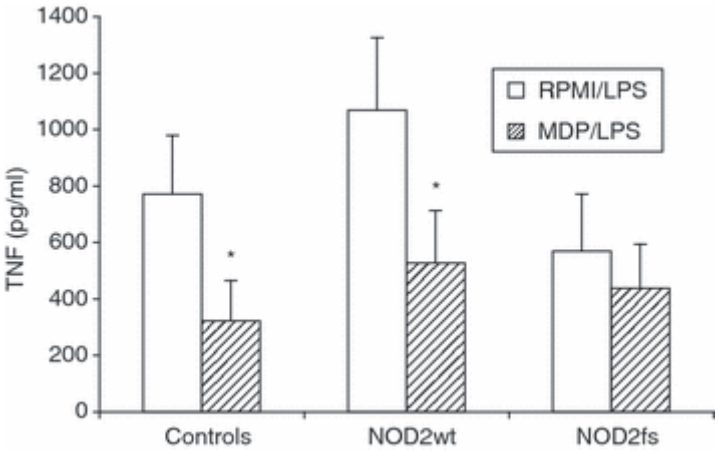

Figure 4. Cross-tolerance induced by Nod2 stimulation is absent in patients with the 3020insC mutation. Cross-tolerance between Nod2 and TLR4, as measured by the effect of MDP (10 μg/ml) preincubation on LPS-induced (1 ng/ml) TNFα production, was assessed in five healthy controls, five Crohn's patients bearing the wild-type allele (Nod2wt), and five patients with the 3020insC Nod2 mutation (Nod2fs). Data are presented as mean ± SD, and compared by Mann–Whitney test ($n = 5$, $^*P < 0.05$).

Discussion

In this study we have demonstrated that stimulation of Nod2 by MDP induces tolerance to subsequent induction of TLR4 by intestinal microorganisms and other ligands. This tolerance affects the production of TNFα, a proinflammatory cytokine with a central role in the pathogenesis of Crohn's disease [19]. Neutralization of TNF by monoclonal antibodies has been proved to be the most effective immunotherapy for Crohn's disease [20]. The observed tolerance may represent an important mechanism allowing the presence of colonizing intestinal flora without activating the immune system. The absence of such tolerization in individuals with Nod2 mutations predisposes to excessive inflammation, and is likely to represent a key factor in the increased susceptibility of these individuals to develop Crohn's disease.

Several mechanisms have been proposed to explain the correlation between loss-of-function mutations in Nod2 and Crohn's disease. We previously proposed that defective synthesis of the anti-inflammatory cytokine IL-10, known to protect against experimental colitis [21], is important for the development of Crohn's disease. Others have proposed that defective barrier defense, through ineffective production of defensins at the level of the intestinal mucosa, could explain bacterial persistence and the induction of inflammation [10,11]. Other hypotheses included Nod2-dependent inhibition of TLR2 signaling [22], or Nod2 3020insC gain-of-function effects on IL-1β release in a mouse model [12], but these two mechanisms could not be confirmed in subsequent studies. Instead, several studies in human primary cells demonstrated synergism between Nod2 and TLR2 stimulation, whereas monocytes isolated from Nod2 3020insC homozygous patients demonstrated defective IL-1β production [9,13,14]. Thus, none of these mechanisms adequately explains the excessive inflammation found in Crohn's disease. Although other pathways may also be involved, we propose that intestinal inflammation in the mucosa of these patients is the result of the absence of Nod2/TLR4 cross-tolerance, leading to subsequent TNFα production induced by intestinal flora triggering TLR4. Although patients with Crohn's disease bearing the Nod2 mutations had a slightly (not significant) lower TNF release compared with the other two groups, they were completely refractory to the Nod2-induced cross-tolerance for LPS stimulation. However, we have to underline that the present study has been performed only with circulating monocytes. Lamina propria monocytes and enterocytes have been reported to have a more tolerant phenotype, and the Nod2/TLR cross-tolerance experiments from the present study should be repeated in lamina propria cells.

Marks et al. recently proposed an intriguing hypothesis for the pathogenesis of Crohn's disease, demonstrating that patients with Crohn's disease have defective production of proinflammatory cytokines, including IL-8, and deficient neutrophil recruitment [23]. This immunodeficient state may lead to ineffective bacterial clearance from the intestinal wall, with subsequent inflammation. However, these defects were reported for Crohn's disease patients irrespective of their Nod2 make-up. Our study presents evidence for a 'second hit'

mechanism in patients bearing Nod2 mutations. Healthy individuals with wild-type Nod2 can mount a proper IL-1β and IL-8 response after challenge with Nod2 ligands [23] enabling elimination of invading organisms, and have an attenuated TNFα release because of the Nod2/TLR4 cross-tolerance. In contrast, patients with Crohn's disease with wild-type Nod2 have an intrinsic IL-8 defect, but this defect can be partly overcome by MDP–Nod2 ligation [23]. However, patients with Crohn's disease and the 3020insC Nod2 mutation have both a deficient IL-8 response, leading to persistence of microbial stimuli, and respond with an abundant TNFα response to intestinal flora because of absence of Nod2/TLR4 cross-tolerance.

The relevance of our data for the pathogenesis of Crohn's is supported by two additional lines of evidence: first, we demonstrated the presence of intracellular peptidoglycans in enterocytes, enabling prolonged interaction with Nod2 and induction of tolerance; and second, tolerance was not only demonstrated for LPS but also for whole intestinal microorganisms. Finally, it should be noted that Nod2 induces specific tolerance for TLR4 stimulation, but not that by TLR2. While both TLR2 and TLR4 mediate nuclear factor-κB translocation and TNF transcription through the myeloid differentiation factor 88 (MyD88)-dependent pathway, in the case of TLR4 (but not TLR2) a second pathway of nuclear factor-κB/TNF induction involves TIR domain-containing adaptor-inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM)-dependent signaling. Thus, one may speculate that it is the TRIF/TRAM TLR4-specific pathway that is modulated by Nod2, although additional experiments are needed to confirm that. In addition, several studies have now shown that TLR4 primarily induces strong proinflammatory signals, whereas TLR2 predominantly provokes an anti-inflammatory profile [24]. Thus, whereas Nod2 induces cross-tolerance against proinflammatory TLR4 signals, it preserves the attenuating TLR2 stimulation. In line with this, only LPS-induced production of TNF, a strong proinflammatory cytokine, has been inhibited by preincubation of cells with MDP, whereas production of the anti-inflammatory cytokines IL-10 and IL-6 was not affected. This demonstrates a specific cross-tolerance effect between Nod2 and TLR4 on TNF release, resulting in a net proinflammatory bias in patients lacking a functional Nod2.

In conclusion, we provide in this study the proof-of-concept that Nod2 can specifically induce cross-tolerance to TNFα stimulation by TLR4 ligands and intestinal microorganisms. This may represent an important mechanism through which overwhelming inflammatory responses to intestinal flora are prevented. However, these effects should be validated in future experiments on lamina propria cells. In addition, additional studies on the precise molecular mechanism of the cross-tolerance are also warranted. This protective cross-tolerance mechanism is absent in patients with Crohn's disease who are homozygous for the 3020insC Npd2 mutation, and this may contribute to their susceptibility to Crohn's disease.

Materials and methods

Patients

Blood was collected from 74 patients with Crohn's disease. Polymerase chain reaction amplification of Nod2 gene fragments containing the polymorphic site 3020insC was performed in 50-µl reaction volumes containing 100–200 ng genomic DNA, as previously described [8]. The 3020insC polymorphism was analysed by Genescan analysis on an ABI Prism 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Five patients identified as homozygous for the 3020insC Nod2 allele were included in the study. From the patients with Crohn's disease who were homozygous for the wild-type Nod2 allele, five were recruited as a control group, in addition to five healthy volunteers with the wild-type allele. None of the patients had active disease at the time of blood donation, and none had used corticosteroids or other anti-inflammatory medication for at least 2 weeks prior to the experiment.

Isolation of mononuclear cells and cross-tolerance

Blood from patients and volunteers was drawn into ethylenediaminetetraacetic acid tubes, and isolation of mononuclear cells was performed as described elsewhere [25]. Mononuclear cells (5×10^5 in 100 µl) were added to round-bottom 96-well plates and preincubated for 24 hr with either 100 µl culture medium (negative control), or MDP at concentrations 0.1, 1 or 10 µg/ml. After incubation for a further 24 hr, the supernatant was discarded, the adherent cells were washed, and restimulation with various stimuli was performed: control medium, highly purified *Salmonella typhimurium* LPS (1 ng/ml), synthetic Pam3Cys or macrophage-activating lipopeptide 2 (MALP) (both at 10 µg/ml), or heat-killed *S. typhimurium* or *Bacteroides fragilis* (both at a concentration of 1×10^6 microorganisms/ml). Enzyme-linked immunosorbent assay was used to measure TNFα, IL-6, IL-10 and IL-12 in the supernatants (Pelikine Compact, Sanquin, the Netherlands).

Immunohistochemistry of intestinal wall peptidoglycans

After obtaining informed consent, colonic biopsies were taken from three patients undergoing routine follow-up colonoscopy for colonic polyps, and three patients with Crohn's disease bearing the wild-type Nod2 allele who were undergoing a routine check-up. Tissue samples were fixed with 4% formaldehyde. After 2 hr the specimens were transferred to a solution of 70% alcohol, and then embedded in paraffin. After dewaxing and dehydration, sections were blocked with normal swine serum followed by 60 min of incubation with a monoclonal mouse anti-MDP antibody (kindly provided by Dr George M. Bahr, Pasteur Institute, Lille, France) at 0.3 µg/ml. The secondary antibody, biotinylated rabbit anti-mouse immunoglobulin, was added to the incubation for 30 min. Slides were stained with streptavidin peroxidase, developed with diaminobenzidine (DAB), and counterstained with haematoxylin for 30 seconds.

Statistical analysis

Experiments were performed in duplicate. Data are given as mean ± SD. The differences between groups were analysed by Mann–Whitney U-test, and where appropriate by paired Wilcoxon test. The level of significance was set at $P < 0.05$.

Acknowledgements

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Reference List

1 Hugot,J.P. *et al.* (1996) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379, 821-823

2 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

3 Hampe,J. *et al.* (2002) Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 359, 1661-1665

4 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

5 Gutierrez,O. *et al.* (2002) Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J. Biol. Chem.* 277, 41701-41705

6 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

7 Inohara,N. *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* 278, 5509-5512

8 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

9 van Heel,D.A. *et al.* (2005) Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 365, 1794-1796

10 Wehkamp,J. *et al.* (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53, 1658-1664

11 Kobayashi,K.S. *et al.* (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307, 731-734

12 Maeda,S. *et al.* (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307, 734-738

13 Netea,M.G. *et al.* (2005) NOD2 3020insC mutation and the pathogenesis of Crohn's disease: impaired IL-1beta production points to a loss-of-function phenotype. *Neth. J. Med.* 63, 305-308

14 Netea,M.G. *et al.* (2005) Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J. Immunol.* 174, 6518-6523

15 Sato,S. *et al.* (2000) Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J. Immunol.* 165, 7096-7101

16 Lehner,M.D. *et al.* (2001) Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. *J. Immunol.* 166, 5161-5167

17 Tapping,R.I. *et al.* (2000) Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. *J. Immunol.* 165, 5780-5787

18 Erridge,C. *et al.* (2004) Lipopolysaccharides of Bacteroides fragilis, Chlamydia trachomatis and Pseudomonas aeruginosa signal via toll-like receptor 2. *J. Med. Microbiol.* 53, 735-740

19 van Deventer,S.J. (1999) Review article: targeting TNF alpha as a key cytokine in the inflammatory processes of Crohn's disease--the mechanisms of action of infliximab. *Aliment. Pharmacol. Ther.* 13 Suppl 4, 3-8

20 Targan,S.R. *et al.* (1997) A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N. Engl. J. Med.* 337, 1029-1035

21 Davidson,N.J. *et al.* (1998) IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J. Immunol.* 161, 3143-3149

22 Watanabe,K. *et al.* (1991) Mechanism for candidacidal activity in macrophages activated by recombinant gamma interferon. *Infect. Immun.* 59, 521-528

23 Marks,D.J. *et al.* (2006) Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet* 367, 668-678

24 Netea,M.G. *et al.* (2005) From the Th1/Th2 paradigm towards a Toll-like receptor/T-helper bias. *Antimicrob. Agents Chemother.* 49, 3991-3996

25 Endres,S. *et al.* (1989) In vitro production of IL 1 beta, IL 1 alpha, TNF and IL2 in healthy subjects: distribution, effect of cyclooxygenase inhibition and evidence of independent gene regulation. *Eur. J. Immunol.* 19, 2327-2333

Chapter 12

The frameshift mutation in Nod2 results in unresponsiveness not only to Nod2- but also Nod1-activating peptidoglycan agonists

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Abstract

NOD2/CARD15 is the first characterized susceptibility gene in Crohn disease. The Nod2 1007fs (Nod2fs) frameshift mutation is the most prevalent in Crohn disease patients. Muramyl dipeptide from bacterial peptidoglycan is the minimal motif detected by Nod2 but not by Nod2fs. Here we investigated the response of human peripheral blood mononuclear cells (PBMCs) from Crohn disease patients not only to muramyl dipeptide but also to several other muramyl peptides. Most unexpectedly, we observed that patients homozygous for the Nod2fs mutation were totally unresponsive to MurNAc-l-Ala-d-Glu-meso-diaminopimelic acid (DAP) (M-TriDAP), the specific agonist of Nod1, and to Gram-negative bacterial peptidoglycan. In contrast, PBMCs from a patient homozygous for the Nod2 R702W mutation, also associated with Crohn disease, displayed normal response to Gram-negative bacterial peptidoglycan. In addition, the blockage of the Nod1/M-TriDAP pathway could be partially overcome by co-stimulation with the Toll-like receptors agonists lipoteichoic acid or lipopolysaccharide. Investigation into the mechanism of this finding revealed that Nod2fs did not act as a dominant-negative molecule for the Nod1/M-TriDAP pathway, implying that the blockage is dependent upon the expression or activity of other factors. We demonstrated that PBMCs from Nod2fs patients express high levels of the peptidoglycan recognition protein S, a secreted protein known to interact with muramyl peptides. We proposed that through a scavenger function, peptidoglycan recognition protein S may dampen M-TriDAP-dependent responses in Nod2fs patients. Together, our results identified a cross-talk between the Nod1 and Nod2 pathways and suggested that down-regulation of Nod1/M-TriDAP pathway may be associated with Crohn disease.

Introduction

Nod2 (also known as CARD15) is a member of the Nod family of pattern recognition molecules involved in peptidoglycan sensing [1,2]. Although Nod2 detects a muramyl dipeptide (MDP)3 motif found in peptidoglycans from all classes of bacteria [3-5], Nod1 detects a diaminopimelic acid (DAP)-containing muramyl tripeptide (M-TriDAP) found primarily in Gram-negative bacterial peptidoglycan [4,6,7]. In addition to its role as an intracellular pattern recognition molecule, genetic evidence has identified Nod2 as the first susceptibility gene for Crohn disease [8,9]. Crohn disease is an inflammatory disorder affecting the digestive tract, the etiology of which remains largely unknown. However, the recent association between the disease and Nod2 on the one hand and between Nod2 and bacterial sensing on the other hand suggests that Crohn disease is likely a consequence of a breakdown in tolerance to the intestinal bacterial flora. Still, it remains unclear why Nod2 dysfunction is a risk factor favoring the onset of Crohn disease. Indeed, although Nod2fs is fully defective for peptidoglycan sensing, other Nod2 mutant proteins found in Crohn disease patients display only minor differences in peptidoglycan detection [10,11].

Through the identification of new important functions of Nod2, substantial progress has been made over the past few years toward understanding the link between Nod2 mutations and Crohn disease [1,2,12]. Indeed, Nod2 function has been shown to be related to intracellular bacterial killing [13], defensin activity due to its expression in Paneth cells [14-16], as well as the induction of the anti-inflammatory cytokine IL-10 [17]. Also, Nod2 –/– mice display an increased TH1 profile of cytokine responses following stimulation with Toll-like receptor (TLRs) agonists [18], which is an observation compatible with some features of Crohn disease. Finally, a recent study has reported the characterization of a knock-in mouse homozygous for the Nod2fs mutation, thus mimicking the natural human mutation associated with Crohn disease [19]. Most surprisingly, macrophages from these animals displayed increased response to MDP, which contradicts results obtained by groups studying human cells [5,17,20]. It is still unclear why Nod2fs mutation seems to represent a loss-of-function in humans and a gain-of-function in the mouse model.

In the present study, we aimed to investigate the response of primary mononuclear cells isolated from Crohn disease patients not only to MDP but also to several muramyl peptides or peptidoglycan agonists. Most surprisingly, we observed that M-TriDAP, the specific agonist of Nod1, failed to induce cytokine response in PBMCs from Nod2fs patients, although it stimulated cells efficiently from either healthy donors or non-Nod2 Crohn disease patients. The importance of this result is further reinforced by the observation that cells from Nod2fs patients were totally unresponsive to peptidoglycan from *Helicobacter pylori*, which is an efficient activator of both Nod1 and Nod2 signaling pathways. Because Nod2fs was unable to act directly as a dominant-negative molecule for the Nod1 pathway, our results rather suggest that the blockage relies on additional factors expressed in Nod2fs cells. Accordingly, we provide evidence that PBMCs from Nod2fs patients express

significantly higher levels of peptidoglycan recognition protein S (PGRP-S) than cells from healthy donors. We propose that PGRP-S expression contributes to the down-regulation of Nod1-dependent responses in Crohn disease patients with Nod2fs mutation. Therefore, these observations suggest the existence of an unexpected cross-talk between the Nod1 and Nod2 signaling pathways. Moreover, our results imply that defects in Nod1 function could participate in the development of Crohn disease. Together, this study provides a basis for the design of original therapeutic approaches for Crohn disease, aiming at establishing a functional Nod1 pathway in Nod2fs patients.

Results and discussion

In the search for MDP-derived muramyl peptides that could stimulate the Nod2 signaling pathway, we generated several molecules differing in the length of their peptidic moiety, including M-TriLys and M-TetraLys (Fig. 1A). These molecules were then tested for their ability to activate Nod2 by using co-transfection assays in HEK293T epithelial cells and measuring NF-κB activity as a read-out (Fig. 1B). By using such tests, we reported previously that the activation of Nod2 was maximal with the addition of 10 pmol of MDP/ml of culture medium (leading to a concentration of 10 nm) [4]. Here a larger range of muramyl peptide concentration was used (10–250 nm) to allow for the identification of even weak inducers of the Nod2 pathway. Through this approach, we observed that MDP and M-TriLys activated Nod2 with similar efficiency, whereas M-TetraLys represented a poor agonist (Fig. 1B). These results are consistent with our previous observations showing that the length of the muramyl peptide stem peptide is a key requirement for induction of Nod2 [4]. Because our goal was to use MDP-derived muramyl peptides to stimulate primary human PBMCs (see below), we searched for other MDP-derived molecules that could represent the optimal negative controls for M-TriLys and M-TetraLys agonists. We took advantage of our previous observation that the sugar moiety of muramyl peptides also plays a key role for optimal activation of Nod2 [4]. We generated modified forms of M-TriLys and M-TetraLys in which the MurNAc moiety is dehydrated to form anhydro-muramyl peptides (see Fig. 1A), and we observed that this subtle modification was sufficient to abolish stimulation of Nod2 (Fig. 1B). Therefore, anhydro-M-TriLys and anhydro-M-TetraLys were subsequently used as control inactive muramyl peptides for M-TriLys and M-TetraLys, respectively. Finally, M-TriDAP, the specific muramyl peptide agonist of Nod1, failed to activate Nod2, even at the highest dose (250 nm) used.

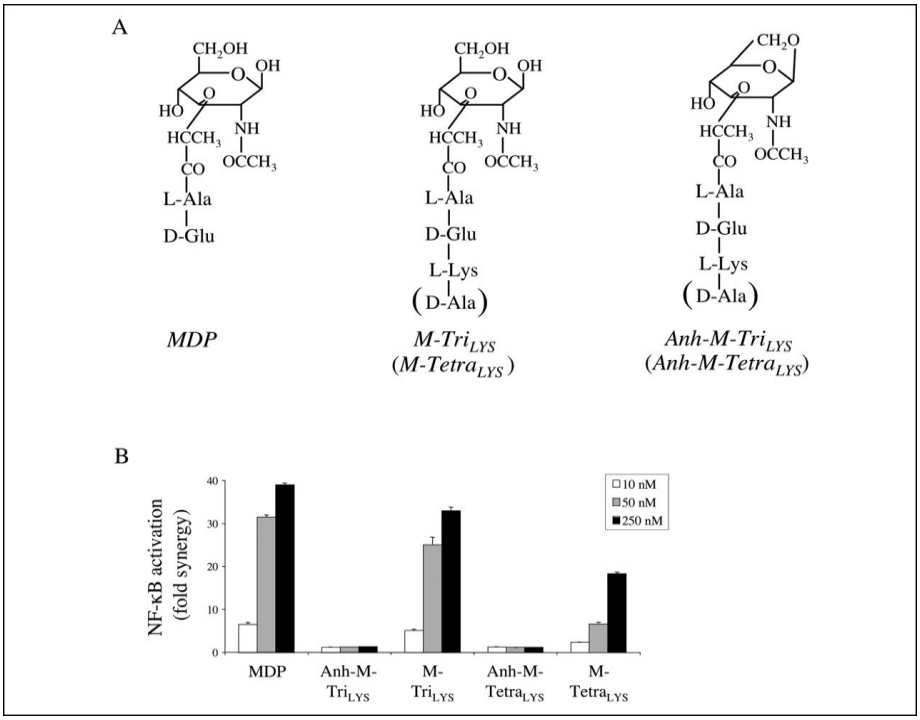


Figure 1. Detection of MDP-derived muramyl peptides by Nod2. A, schematic representation of the muramyl peptides used in this study. B, human HEK293 epithelial cells were transfected with several muramyl peptides (MDP, M-TriLYS, Anh-M-TriLYS, M-TetraLYS, and Anh-M-TetraLYS) at the following concentrations: 10 nm (white bars), 50 nm (gray bars), or 250 nm (black bars) in the presence of an expression vector for Nod2. The activity of an NF-κB-driven luciferase reporter gene was measured, and Nod2-dependent activation of the reporter gene in the presence of muramyl peptides was reported to the one obtained without stimulation with muramyl peptides. Data show the mean ± S.E. of duplicate experiments. Experiments were performed three times with similar results

The six muramyl peptides characterized above (MDP, M-TriLys, M-TetraLys, anhydro-M-TriLys, anhydro-M-TetraLys, and M-TriDAP) were used to stimulate PBMCs obtained from human blood. PBMCs from three groups of individuals were collected: healthy donors (control, CTR), Crohn disease patients without Nod2 mutations (Crohn), and Crohn disease patients carrying homozygous Nod2fs frameshift mutation (Nod2fs). Muramyl peptides were directly added to the culture medium at a final concentration of 50 nm, and supernatants were collected following overnight stimulation. IL-1β, IL-10, and TNFα were measured in the supernatant, whereas intracellular IL-1α was measured from cell lysates (Fig. 2A). First, our results identified MDP and M-TriLys as potent activators of human PBMC responses, and we confirmed that the detection of these muramyl peptides depends upon Nod2 because Nod2fs cells were not stimulated by MDP and M-TriLys (Fig. 2A). In addition, our observation that M-TetraLys was a poor inducer of Nod2 in vitro was reinforced by our findings that this agonist only marginally induced cytokine secretion from human PBMCs, and that this effect was further blunted in cells from Nod2fs patients (Fig. 2A). More importantly, our results showed that Nod2-independent Crohn disease patients still reacted to MDP and M-TriLys, thus demonstrating that the inability of cells from Nod2fs patients to detect these agonists resulted from their Nod2 mutation and was not an indirect consequence of the disease. Consequently, this observation also suggested that defects in muramyl peptide sensing are not the sole cause of Crohn disease development. Second, the conclusion that sensing of M-TriLys in PBMCs from healthy donors and non-Nod2 Crohn disease patients depends on Nod2 is reinforced by the observation that anhydro-M-TriLys failed to stimulate these cells, which is in agreement with the results obtained in HEK293T cells (see Fig. 1B). Finally, we aimed to use M-TriDAP in order to stimulate PBMCs in a Nod1-dependent but Nod2-independent manner. M-TriDAP stimulation induced cytokine secretion in human PBMCs from healthy donors or non-Nod2 Crohn disease patients (Fig. 2A). However, M-TriDAP was strikingly unable to stimulate PBMCs from Nod2fs patients. This result was unexpected because M-TriDAP is a specific activator of Nod1 but not of Nod2. Therefore, these results identified an unexpected link between Nod2 mutations and the Nod1 signaling pathway.

Muramyl peptides are naturally occurring degradation products of peptidoglycan, which are useful tools to study precisely the involvement of signaling pathways dependent upon the specific activation of Nod1 or Nod2. However, in physiological situations, macrophages would likely encounter the presence of both intact peptidoglycan polymers together with muramyl peptides. Therefore, we aimed to investigate the response of PBMCs from the

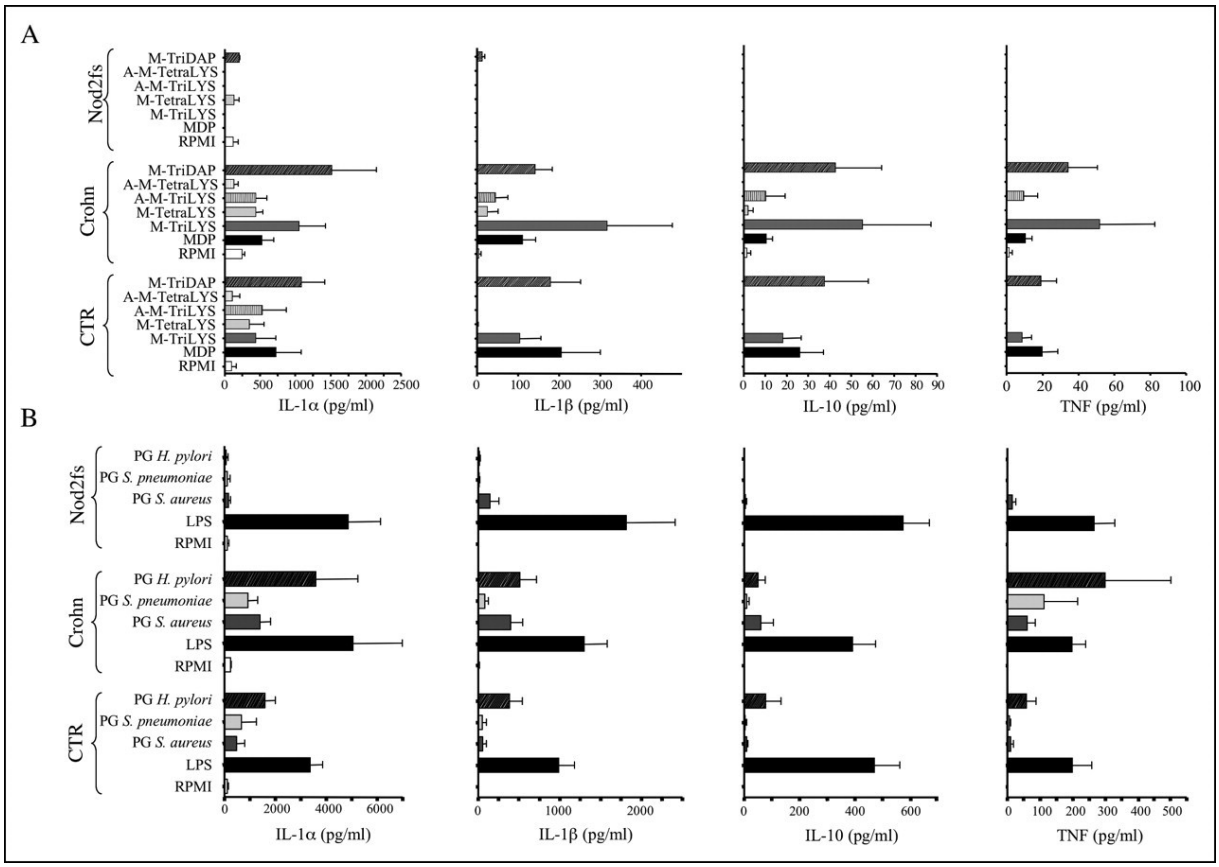


Figure 2. Response to muramyl peptides and peptidoglycans of PBMCs from healthy donors (CTR; n = 5), Crohn disease patients without defects in Nod2 (Crohn; n = 5), or Crohn disease patients homozygous for the frameshift mutation (Nod2fs; n = 4). A and B, IL-1β, TNF, and IL-10 were measured from the cell culture medium. Intracellular IL-1α was measured from cell lysates. Muramyl peptides (A, all 50 nm) or peptidoglycans (B, all peptidoglycans at 10 µg/µl; LPS (100 ng/ml)) were added directly to the cell culture medium for 18 h.

same individuals to peptidoglycans from *H. pylori*, *S. pneumoniae*, and *S. aureus*. We decided to use peptidoglycan from *H. pylori* because it is the prototype of Gram-negative bacterial peptidoglycan (DAP-type peptidoglycan) and is relatively easy to purify. Similarly, peptidoglycan from *S. pneumoniae* was chosen because it represents a classical peptidoglycan (Lys-type peptidoglycan) from Gram-positive bacteria. Finally, peptidoglycan from *S. aureus* was also included in this study because it is widely studied; however, because of the extremely high degree of peptidic cross-linking found in this peptidoglycan, its structure is less representative of Gram-positive bacterial peptidoglycan than that of *S. pneumoniae*. For such studies, the level of purification of the peptidoglycan polymer is a crucial feature. Several quality control tests were performed along the purification steps to ensure that other cell wall contaminants are excluded, such as LPS, lipoproteins, or LTA. To this end, the absence of LPS contamination was assessed by the Limulus amebocyte lysate test, showing that purified peptidoglycans contained less than 4 pg of LPS/ml of sample (data not shown). To address the difficult question of contamination by lipoproteins or LTA, we took advantage of our recent observation that contaminant-free peptidoglycans failed to stimulate thioglycollate-induced peritoneal macrophages from mice [21]. Our purified

peptidoglycans failed to induce the secretion of TNFα or IL-6 from peritoneal mouse macrophages (data not shown), showing that only traces amounts, if any, of lipoproteins or LTA contaminants were present in our peptidoglycan preparations. These peptidoglycan preparations were then added (each at 10 μg/ml) to the human PBMCs from the same individuals as described above, and the cytokines were measured after overnight stimulation (Fig. 2B). As a control, cells were also stimulated with LPS (100 ng/ml). First, by analyzing the cytokine response of cells from the healthy donors, we noticed that purified peptidoglycans activate human PBMCs, which contrasts with the lack of response in mouse peritoneal macrophages (see above). The reason for this discrepancy remains unknown, but it strongly correlates with the blunted response of mouse macrophages to muramyl peptides.4 Second, we observed that cells from non-Nod2 Crohn disease patients also responded to peptidoglycans as well (or even slightly more, depending on the cytokines) as the healthy donors (Fig. 2B). Finally, we found that PBMCs from the Nod2fs group of patients were totally unresponsive to the three peptidoglycans used in this study, regardless of the cytokine analyzed. More importantly, these cells were still fully responsive to LPS stimulation, thus demonstrating that Nod2fs PBMCs did not display a global unresponsiveness to any stimulation. Again, these results are in agreement with the data from cells stimulated with muramyl peptides (see Fig. 2A).

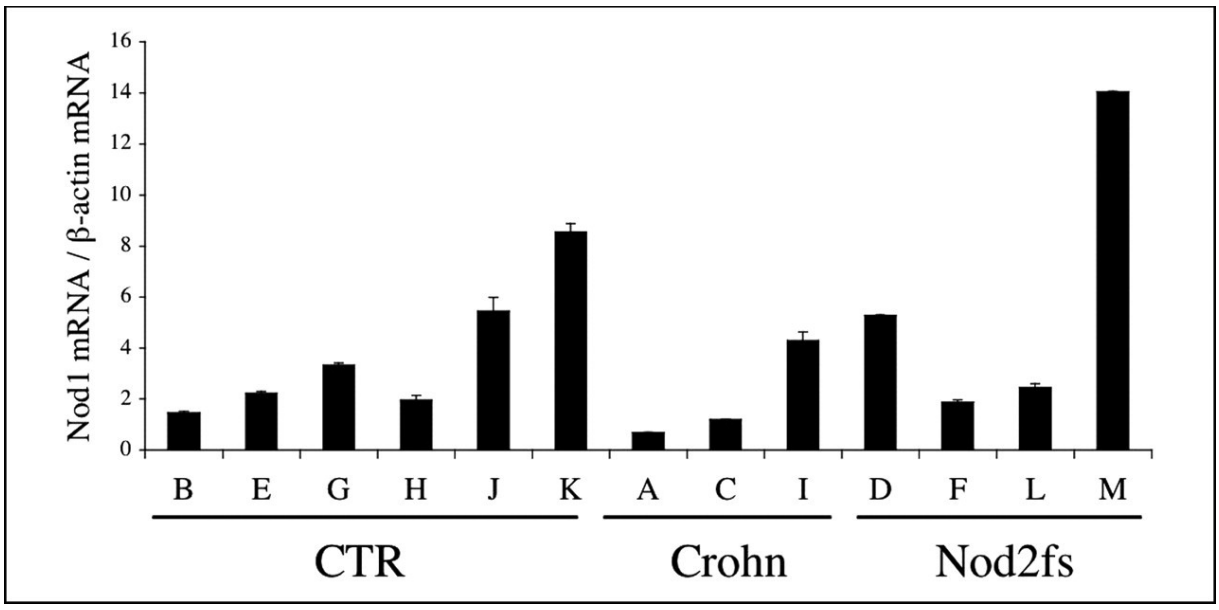


Figure 3. Expression of Nod1 in PBMCs from 13 individuals by real time PCR. Expression of Nod1 was analyzed by real time PCR in cells from healthy donors (CTR), Crohn disease patients without defects in Nod2 (Crohn), or Crohn disease patients homozygous for the frameshift mutation (Nod2fs). Expression of Nod1 is reported to the internal control β-actin.

Our observation that PBMCs from Nod2fs patients are unresponsive to Gram-positive bacterial peptidoglycans allows us to draw some important conclusions. First, this result shows that Nod2 is a key sensor of the Gram-positive bacterial peptidoglycan. Second, this observation suggests that, within the peptidoglycan polymer, the MDP and M-TriLys motifs are the key structures that drive the response of the host through their detection by Nod2.

Third, our assumption that these peptidoglycans are free of bacterial contaminants is reinforced by this result, because unpurified peptidoglycans would have induced a TLR-driven response. In the case of the Gram-negative bacterial peptidoglycan, it was anticipated that, in the cells from Nod2fs patients, the defective Nod2 sensing could be compensated by the activation of the Nod1 signaling pathway. Indeed, unlike Gram-positive bacterial peptidoglycan, Gram-negative bacterial peptidoglycan is able to stimulate both Nod1 and Nod2. The lack of Nod1-dependent signaling in Nod2fs cells (Fig. 2B) again suggests that a functional Nod2 signaling pathway is required for Nod1-driven signaling to take place. This result confirms and extends the conclusions from the study of muramyl peptide stimulation of PBMCs (see Fig. 2A). Taken together, it can be concluded that any peptidoglycan sensing (dependent upon Nod2, Nod1, or any uncharacterized peptidoglycan sensor) is abrogated in PBMCs from Nod2fs patients. This defect is not found in non-Nod2 Crohn disease patients, therefore suggesting that if lack of peptidoglycan sensing contributes to the onset of Crohn disease, the pathology can also arise from other causes.

In an attempt to better understand the origin of the defective Nod1-dependent signaling in cells from Nod2fs patients, we aimed to define whether such a defect was specific to the Nod2fs mutation. We took advantage that one individual in our cohort of Crohn disease patients had been genotyped as homozygous for the Nod2 R702W mutation, which is the second most frequent Nod2 mutation associated with increased risk for Crohn disease. Most strikingly, PBMCs from this patient were responsive to *H. pylori* peptidoglycan to a similar extent as the control group (TABLE ONE), which contrasts with the results obtained from Nod2fs patients. Obviously, these results will need further confirmation by analyzing larger populations of patients carrying diverse Nod2 mutation; still, this initial characterization suggests that the defect in the Nod1/M-TriDAP pathway that we have identified is likely to be associated with the Nod2fs genotype and not other Nod2 mutations.

	CTR		
	RPMI	LPS	PG *H. pylori*
TNF	0	198 ± 47	58 ± 25
IL-1β	0	987 ± 169	382 ± 152
IL-10	0	470 ± 87	78 ± 44
IL-1α	103 ± 51	3368 ± 319	1580 ± 287
	Nod2fs		
	RPMI	LPS	PG *H. pylori*
TNF	0	267 ± 54	0
IL-1β	0	1815 ± 447	14 ± 7
IL-10	0	573 ± 66	0
IL-1α	127 ± 42	4860 ± 823	75 ± 50
	Nod2 R702W		
	RPMI	LPS	PG *H. pylori*
TNF	0	222	60
IL-1β	0	1085	665
IL-10	3	656	72
IL-1α	265	3560	2700

Table 1 Response of PBMCs from a Crohn disease patient homozygous for the R702W mutation to *H. pylori* PG. IL-1β, TNF, and IL-10 were measured from the cell culture medium. Intracellular IL-1α was measured from cell lysates. *H. pylori* peptidoglycan (10 µg/ml) and LPS (100 ng/ml) were added directly to the cell culture medium for 18 h. Values given represent pg/ml of cytokine measured ± S.E.

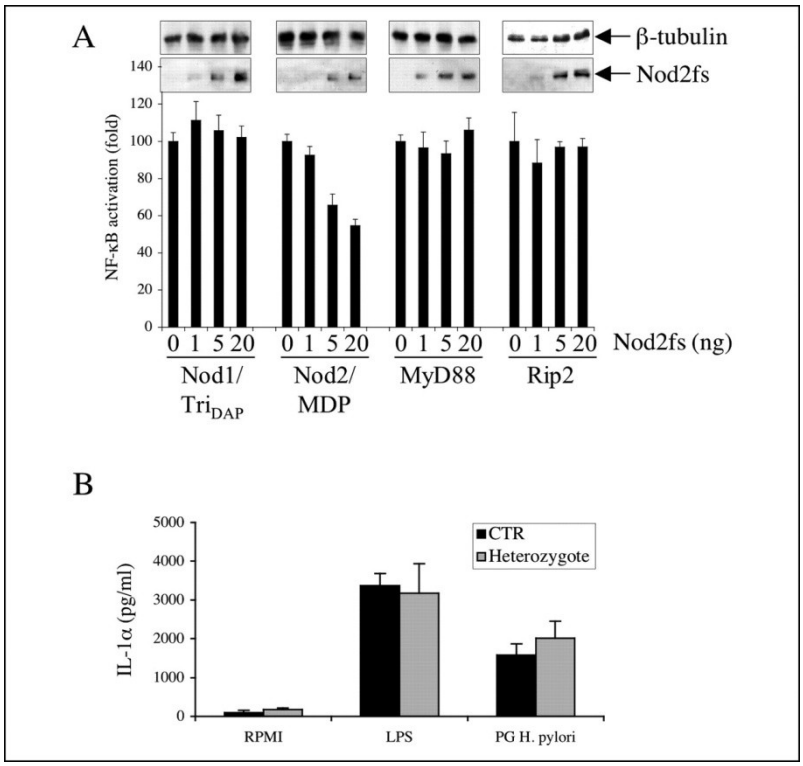


Figure 4.Nod2fs does not act as a dominant-negative molecule on the Nod1/M-TriDAP pathway. A, human HEK293 epithelial cells were transfected with several constructs that trigger the NF-κB pathway: Nod1 (1 ng of DNA added; simultaneously, addition of 50 nm M-TriDAP); Nod2 (0.5 ng of DNA added; simultaneously, addition of 50 nm MDP); Rip2 (50 ng); MyD88 (1 ng). Co-transfection of the above constructs was then performed together with increasing amounts (1, 5, and 20 ng) of expression vector for Nod2fs, and the activity of a NF-κB-driven luciferase reporter gene was measured. NF-κB activations are presented as a percentage of the value obtained in the absence of Nod2fs expression. Data show the mean ± S.E. of duplicate experiments. Experiments were performed three times with similar results. B, the response of PBMCs from healthy donors (CTR; n = 4) and Crohn disease patients heterozygous for the Nod2fs mutation (heterozygote; n = 4) to H. pylori peptidoglycan (PG) was analyzed. Intracellular IL-1α was measured from cell lysates. H. pylori peptidoglycan (10 μg/μl) and LPS (100 ng/ml) were added directly to the cell culture medium for 18 h.

Next, we investigated whether Nod1 expression was decreased in Nod2fs PBMCs. Nod1 expression was analyzed by real time PCR on 13 individuals (6 “CTR”, 3 “Crohn,” and 4 “Nod2fs”). Even though expression of Nod1 was found quite variable among individuals, no correlation could be observed between expression levels of Nod1 and the three groups analyzed (Fig. 3). Therefore, the lack of the Nod1-dependent response in Nod2fs PBMCs cannot be accounted for by a defect in Nod1 expression in these cells. One likely explanation for the defective Nod1/M-TriDAP pathway in cells from Nod2fs patients is that the Nod2fs protein would simply behave as a dominant-negative for Nod1 signaling. To test this hypothesis, HEK293 cells were transfected with Nod1 plus M-TriDAP (1 ng of Nod1; 50 nm M-TriDAP; according to standard procedures [4]) in the presence of increasing amounts of Nod2fs. Clearly, Nod2fs was unable to block the Nod1/M-TriDAP pathway (Fig. 4A), even at the highest dose tested (50 ng of Nod2fs DNA transfected). Also, we found no effect of

Nod2fs overexpression on NF-κB activation triggered by either Rip2 or MyD88 (Fig. 4A), whereas the molecule blocked only partially the NF-κB activation induced by Nod2/MDP (–45%). Therefore, in light of these results, one can rule out a direct effect of Nod2fs on Nod1 signaling. Accordingly, if Nod2fs protein had behaved as a dominant-negative molecule on the Nod1 pathway, it is likely that patients heterozygous for the Nod2fs mutation would have displayed altered sensing of *H. pylori* peptidoglycan. This was indeed not the case (Fig. 4B; similar results were obtained with the other cytokines measured (data not shown)).

Next, we analyzed whether the lack of response of Nod2fs cells to M-TriDAP was still observed in the case of co-stimulation with other agonists. Indeed, it is well characterized that muramyl peptides act in synergy with TLR agonists to induce cytokine secretion from PBMCs [22-26]. Therefore, we stimulated PBMCs from individuals in our three groups (CTR, Crohn, and Nod2fs) with M-TriDAP, LPS (TLR4 agonist), or LTA (TLR2 agonist) either alone or in combination (M-TriDAP + LPS or M-TriDAP + LTA). For the three groups of individuals, we observed that M-TriDAP could function in synergy with LPS or LTA to potentiate cytokine secretion (Fig. 5; similar results were obtained with the other cytokines measured (data not shown)). These results are in agreement with the recent evidence presented in two independent studies, in which PBMCs from healthy donors were used [27,28]. As a control, PBMCs were also stimulated with MDP in combination with LPS or LTA. As reported previously [17], synergistic induction of cytokine secretion was observed in the case of healthy individuals or non-Nod2 Crohn disease patients but not in the Nod2fs group (Fig. 5). Together, these results suggest that even though M-TriDAP does not directly induce cytokine secretion in Nod2fs PBMCs, the blockage can be partially overcome in the case of co-stimulation with TLR ligands.

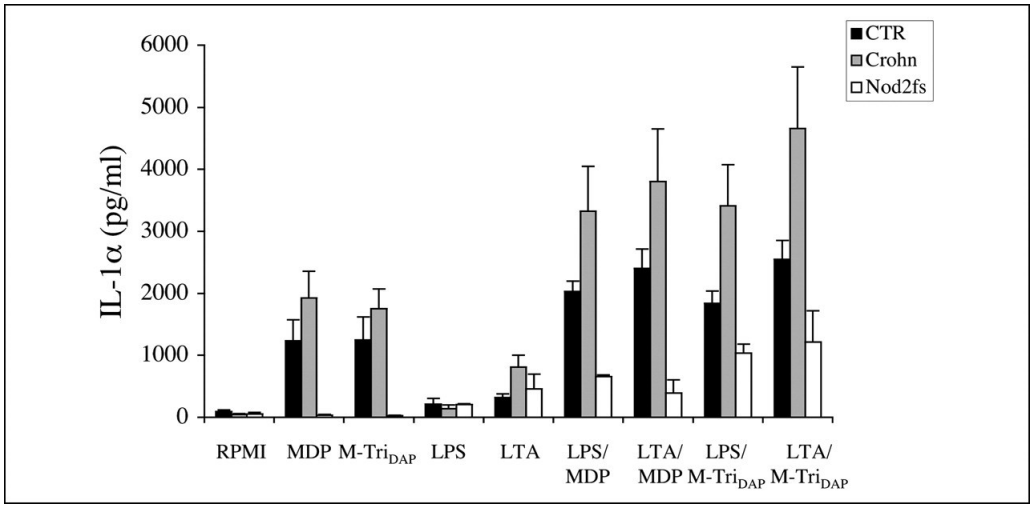


Figure 5. Synergistic activation of PBMCs by MDP or M-TriDAP plus either LPS or LTA. PBMCs from healthy donors (CTR; n = 6), Crohn disease patients without defects in Nod2 (Crohn; n = 3) or Crohn disease patients homozygous for the frameshift mutation (Nod2fs; n = 4) were analyzed. Cells were stimulated either with MDP or M-TriDAP, LPS, or LTA alone or in combination: LPS + MDP, LTA + MDP, LPS + M-TriDAP, or LTA + M-TriDAP with the agonists added simultaneously. Intracellular IL-1α was measured from cell lysates. LPS (1 ng/ml), LTA (5 µg/ml), MDP (50 nm), and M-TriDAP (50 nm) were added directly to the cell culture medium for 18 h.

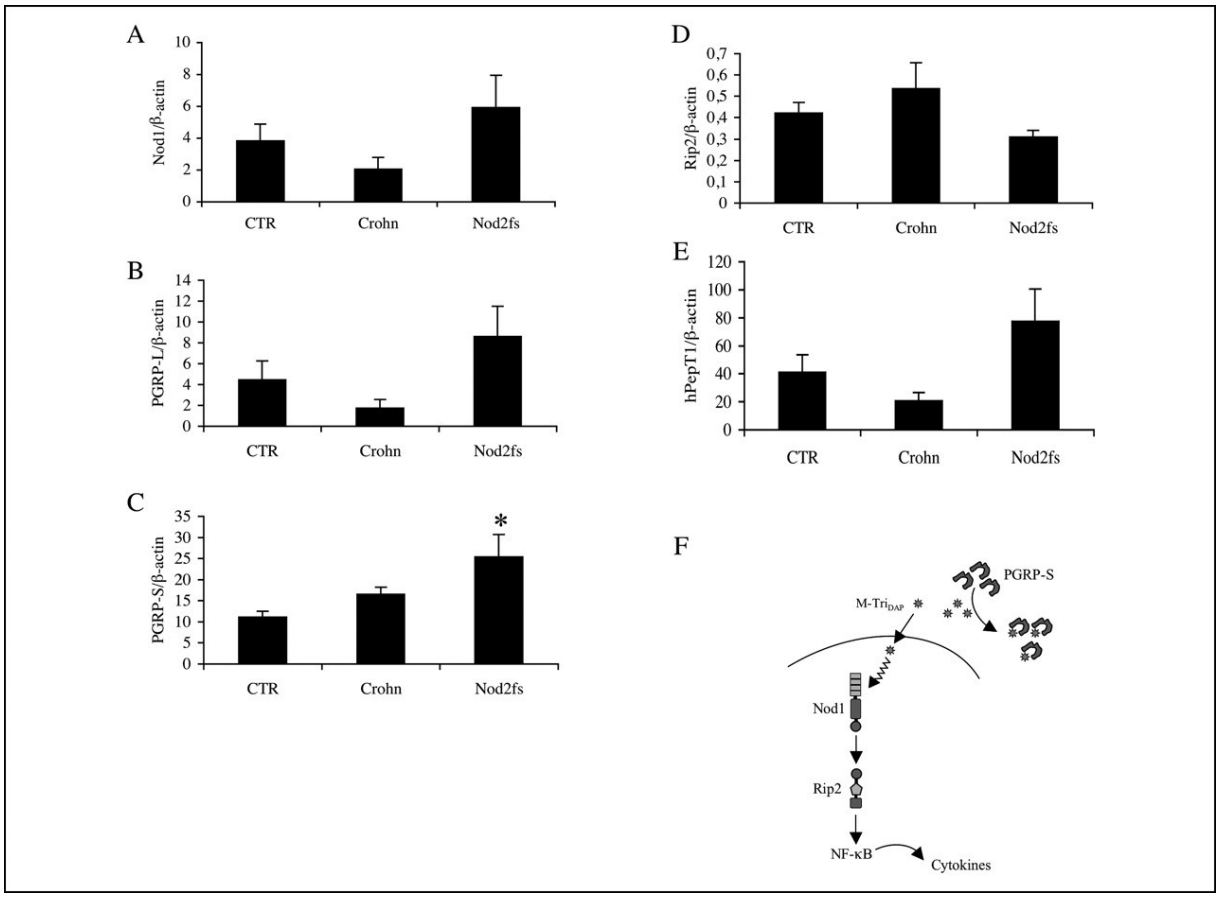


Figure 6. Expression of Nod1, PGRP-L, PGRP-S, Rip2, and hPepT1 in PBMCs from 13 individuals by real time PCR. A–E, expressions of Nod1 (A), PGRP-L (B), PGRP-S (C), Rip2 (D), and hPepT1 (E) were analyzed by real time PCR in cells from healthy donors (CTR), Crohn disease patients without defects in Nod2 (Crohn), or Crohn disease patients homozygous for the frameshift mutation (Nod2fs). In each case, expression of the gene of interest is reported to the internal control β-actin. Asterisk indicates that the difference of expression of PGRP-S between the control and Nod2fs groups has been found as statistically significant using the Mann-Whitney test ($p < 0.05$). F, schematic model illustrating the possible role of PGRP-S as a scavenger molecule for muramyl peptides in the extracellular compartment of PBMCs. An up-regulation of PGRP-S (as observed in Nod2fs patients) would favor the titration of muramyl peptides (including M-TriDAP) out of the activation pathway.

In order to gain more insights into the mechanism responsible for the blockage of the Nod1 pathway in Nod2fs cells, real time PCR analysis of selected candidate genes was performed in PBMCs isolated from our three groups of individuals (6 healthy donors (CTR), 3 Crohn disease patients without mutations in the Nod2 gene (Crohn), and 4 Nod2fs patients). The selected genes were the following and were chosen based on their potential roles in muramyl peptide signaling: PGRP-L, PGRP-S, RIP2, and hPEPT1 (Nod1 expression was also presented as a comparison). Indeed, PGRP-L and PGRP-S both interact with muramyl peptides and peptidoglycan [29-31]. Rip2 is a crucial adaptor protein downstream of both Nod1 and Nod2 pathways [32-37]. Finally, hPepT1 has been demonstrated to allow for the internalization of muramyl dipeptide, at least in epithelial cells [38]. Even though differences in expression were noted for these genes among the groups tested (Fig. 6, A–E), all except one

case, these variations were not judged statistically significant (95% confidence) using the Mann-Whitney test for nonparametric samples. The only strong correlation was found in the Nod2fs group, in which a significant increase of PGRP-S expression was observed as compared with the control group of healthy donors (Fig. 6C; $p < 0.05$). Because PGRP-S is a secreted protein known to bind muramyl peptides, one can speculate that excessive expression of PGRP-S by Nod2fs PBMCs might contribute to dampening the response of these cells to muramyl peptides, thereby acting as a scavenger molecule (Fig. 6F). In this context, it is interesting to note that PGRP-SC1B, a Drosophila homolog of mammalian PGRP-S, has been proposed to modulate the innate immunity in the fruit fly also via a scavenger function [39]. Further investigation will be required to define precisely the role of PGRP-S in muramyl peptide-dependent signaling in both normal and inflammatory conditions.

The defective Nod1 function in PBMCs from Nod2fs patients was, at least in part, overcome when cells were co-stimulated with TLR ligands, such as LPS or LTA (see Fig. 5). This observation strongly suggests that in cells expressing functional TLRs, the defective Nod1 pathway may not have a crucial impact on the etiology of Crohn disease. However, this defect could prove of critical importance in epithelial cells lining the mucosal surfaces. Indeed, these cells are permanently in contact with microbes and microbial products, and therefore down-regulation of TLR function represents a common mechanism to avoid constitutive inflammation due to the microbial flora [40]. Accordingly, by using ex vivo experiments, we have been able to show that intestinal epithelial cells detect nonflagellated bacteria exclusively through Nod1 (6). As a consequence, it can be envisioned that defective function of Nod1 in intestinal epithelial cells from Nod2fs patients may participate in the establishment of Crohn disease. This hypothesis has recently found strong support through the identification of genetic variation in Nod1 associated with predisposition to inflammatory bowel disease [41].

The results presented here may have an impact on the design of new therapeutic treatments for Crohn disease. Nod2 1007fs mutation represents one-third to one-half of the Nod2 mutations found in Crohn disease patients. In this group of patients, a therapeutic approach aimed at restoring functional Nod1 signaling can be envisioned. Indeed, up until now, the idea of targeting Nod1 pathway in Crohn disease patients was not envisioned in this way, because it was assumed that Nod1 remained fully functional. Restoring a functional Nod1 pathway in Nod2fs cells would have the important advantage of restoring partial homeostasis of the intestinal mucosa vis à vis the microbial environment. Therefore, if Crohn disease is a consequence of a breakdown in the tolerance to the intestinal bacterial flora, such tolerance could be restored through Nod1-dependent sensing of Gram-negative bacterial components of the microbial environment. Because such therapy would rely on a fine balance defined by the host itself, it would be likely less aggressive than other treatments, such as those acting to reduce the inflammation induced by the disease. However, one can question whether restoring the sensing of Gram-negative bacterial species only is sufficient to promote intestinal homeostasis to the microbial environment. This issue is of crucial importance for both our fundamental understanding of the etiology of Crohn disease and the rational design of new therapeutics aimed at treating the pathology.

Materials and methods

Preparation of highly purified peptidoglycans from Gram-negative and Gram-positive bacteria

Bacterial strains used to prepare peptidoglycans are the following: *H. pylori* 26695; Staphylococcus aureus COL (from Olivier Chesneau, Institut Pasteur); and *Streptococcus pneumoniae* R800. The peptidoglycans purification procedures were exactly as described previously [6,21]. Purity of samples was assessed by HPLC amino acid and saccharide analysis after HCl hydrolysis. Also, each peptidoglycan preparation was tested for the absence of LPS contamination using the Limulus amebocyte lysate assay as described previously [21]. The absence of TLR2-detected contaminants (lipoproteins or lipoteichoic acids) was tested on thioglycollate-elicited mouse peritoneal macrophages from either C57Bl6 or TLR2–/– mice as described previously [21].

Preparation of muropeptides

DAP- and Lys-containing UDP-Mur-NAc-peptides were prepared as described previously [4,42]. M-TetraLYS, M-TriLYS, and M-TriDAP were generated by mild acid hydrolysis (0.1 m HCl, 10 min at 100 °C) of the corresponding UDP-MurNAc peptides. Replacement of meso-DAP by l-Lys in the peptidoglycan of *Escherichia coli* was obtained by overexpression in the latter species of the murE gene from *S. aureus* encoding UDP-MurNAc-l-Ala-d-Glu:l-Lys adding enzyme [27]. Cells were harvested before cell lysis occurred, and their peptidoglycan was extracted and purified as described previously [28]. In these conditions, about 50% of the DAP residues at the third position of the peptides was shown to be replaced by l-Lys. This peptidoglycan preparation was digested by SltY lytic transglycosylase in a reaction mixture (1 ml) consisting of 300 mm sodium acetate buffer, pH 4.5, 1 mg of PG (briefly sonicated for homogenization), and 100 µgof purified SltY enzyme [43]. After overnight incubation at 37 °C, the reaction was stopped by adding 500 µl of 50 mm sodium phosphate buffer, pH 4.45 (HPLC eluent A), and 2 µl of phosphoric acid. The two main monomer products, Anh-GM-TetraDAP and Anh-GM-TetraLYS, were purified by HPLC on a column of nucleosyl 5C18 (4,6 × 250 mm, Alltech). Elution was performed at 0.6 ml/min with buffer A, using a gradient of methanol from 0 to 25% for 180 min. Detection was at 215 nm. The retention times of these two compounds were 67 and 80 min, respectively. They were further purified and desalted using a second HPLC step on the same column but this time using 0.1% trifluoroacetic acid and a gradient of methanol for elution. Their purity and composition were confirmed by amino acid and hexosamine analysis after acid hydrolysis of samples (6 m HCl, 16 h at 95 °C), using an Hitachi L8800 analyzer, as well as by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Anh-M-TetraLYS was obtained by treatment of Anh-GM-TetraLYS with *E. coli* NagZ β N-acetylglucosaminidase. The reaction mixture (200 µl) contained 20 mm HEPES buffer, pH 7.4, 50 mm NaCl, 0.5 mm substrate, and 20 µg of purified NagZ enzyme [43]. Anh-GM-TriLYS and Anh-M-TriLYS were generated by treatment of the corresponding tetrapeptide compounds with *E. coli* LdcA ld-carboxypeptidase. The reaction mixture (200 µl) contained 50 mm Tris-HCl buffer, pH 8.0, 0.5 mm substrate, and 20 µg of

purified LdcA enzyme [43]. In all cases, incubation was for overnight at 37 °C, and the products were purified by HPLC, and their identity was confirmed by the above described procedures.

Cell lines and reagents

HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Prior to transfection, HEK293T cells were seeded into 24-well plates at a density of 1 × 10^5 cells/ml as described previously [32]. MDP LD (l-alanine d-glutamine) was from Calbiochem and reported to be 98% pure by TLC. Highly purified *E. coli* LPS (strain O55:B5) was from Invitrogen. Artificially synthesized LTA was kindly provided by Dr. Corinna Hermann (Konstanz University, Germany). Anti-Nod2 rabbit polyclonal antibody was from Cayman Chemical (Ann Harbor, MI), and anti-β-tubulin monoclonal antibody was from Sigma.

Expression plasmids and transient transfections

The expression plasmids for Nod1 and Nod2 were the kind gift from Gabriel Nuñez (University of Michigan Medical School, Ann Arbor, MI) and have been described previously [44]. The expression vector for Nod2fs was from M. Giovannini (INSERM U674, CEPH, Paris, France). Constructs for Rip2 and MyD88 were kind gifts from M. Thome (ISREC, Lausanne, Switzerland) and M. Muzio (Mario Negri Institute, Milan, Italy), respectively. The NF-κB luciferase reporter plasmid was from Stratagene. Transfections and Western blotting experiments were carried out in HEK293T cells as described previously [32].

NF-κB activation assays

Studies examining the synergistic activation of NF-κB by muramyl peptides in cells overexpressing Nod2 were carried out as originally described by Inohara et al. [44]. Briefly, HEK293T cells were transfected overnight with the expression vectors of interest plus 75 ng of NF-κB luciferase reporter plasmid. At the same time, muramyl peptides were added to the cell culture medium, and the synergistic NF-κB-dependent luciferase activation was then measured following 24 h of co-incubation. NF-κB-dependent luciferase assays were performed in duplicate, and the data represent at least three independent experiments. Data show means ± S.E.

Genotyping of Nod2 variants

Blood was collected from 74 patients with Crohn disease and 10 healthy volunteers. PCR amplification of Nod2 gene fragments containing the polymorphic sites 3020insC (for Nod2fs) and C2104T (for R702W) was performed in 50-µl reaction volumes containing 100–200 ng of genomic DNA, as described previously [17]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI Prism 3100 genetic analyzer according to the protocol of the manufacturer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands).

Four patients with Crohn disease were found homozygous for the 3020insC mutation; one patient with Crohn disease was found homozygous for the C2104T mutation, and they were

further investigated in the cytokine studies. As control groups, five patients with Crohn disease heterozygous for the 3020 insC Nod2 mutation, five patients with Crohn disease bearing the wild type allele, and five healthy volunteers homozygous for the wild type Nod2 allele were included. The cells isolated from the four groups of patients were isolated and tested at two separate occasions. The study was approved by the Ethical Committee of the Radboud University, Nijmegen, The Netherlands.

Isolation of mononuclear cells and stimulation of cytokine production

After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10-ml EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Amersham Biosciences). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10 μg/ml gentamicin, 10 mml-glutamine, and 10 mm pyruvate. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number was adjusted to 5×10^6 cells/ml. 5×10^5 PBMCs in a 100-μl volume were added to round-bottom 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) and were incubated with either 100 μl of culture medium (negative control) or the following various stimuli: 50 nm of the various muropeptide preparations, 10 μg/ml of the purified peptidoglycans, 100 ng/ml highly purified *E. coli* LPS (strain O55:B5), 5 μg/ml of artificially synthesized LTA (kindly provided by Dr. Corinna Hermann, Konstanz University, Germany), or a combination of stimuli as described under “Results and Discussion.”

Cytokine measurements

For detection of cytokine concentrations in the supernatants, BioPlex 100 system (Bio-Rad) was used. The kits were used as indicated by the manufacturer, and the sensitivity for all cytokines was <20 pg/ml.

Real time PCR studies

Total RNA was isolated from cells using RNeasy kits (Macherey Nagel, Hoerdt, France) according to the manufacturer's instructions. RNA quantification was performed using spectrophotometry. After treatment at 37 °C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics), oligo(dT) primers (Roche Diagnostics) were used to synthesize single-stranded cDNA. mRNAs were quantified using SYBR green master mix (Applera, Courtaboeuf, France) with specific human oligonucleotides in a GeneAmp Abiprism 7000 (Applera, Courtaboeuf, France). The following primers were used: Nod1, sense GTAAAGGTGCTAAGCGAAGA and antisense TCTGATTCTGGATAAGCCAT; hPepT1, sense CCGCCTCCCAGGTTCAA and antisense GGTGCATGCCGCTAATCC; PGRP-S, sense GCAGCACTACCACATGAAGACACT and antisense GAGCCCGTCTTCTCCAATCA; PGRP-L, sense ACTGAGGGCTGCTGGGACCA and antisense GGCCTCAGTGAATTCCTTGG; Rip2, sense AAATGGATCATTAAATGAACTCCTACATAG and antisense TTCATGCAGGATGCGAAATC. In each assay, calibrated and no-template controls were included. Each sample was run in duplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applera,

Courtaboeuf, France). All results were normalized to the β-actin, an unaffected housekeeping gene.

Statistical analysis

The human experiments were performed in triplicate with blood obtained from patients and volunteers. The differences between groups were analyzed by the Mann-Whitney U test and where appropriate by the Kruskal-Wallis analysis of variance test. The level of significance between groups was set at $p < 0.05$. The data are given as means ± S.E.

Reference List

1 Girardin,S.E. *et al.* (2003) Lessons from Nod2 studies: towards a link between Crohn's disease and bacterial sensing. *Trends Immunol.* 24, 652-658

2 Inohara *et al.* (2005) NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 74, 355-383

3 Girardin,S.E. *et al.* (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869-8872

4 Girardin,S.E. *et al.* (2003) Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J. Biol. Chem.* 278, 41702-41708

5 Inohara,N. *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* 278, 5509-5512

6 Girardin,S.E. *et al.* (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

7 Chamaillard,M. *et al.* (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* 4, 702-707

8 Hugot,J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599-603

9 Ogura,Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603-606

10 Chamaillard,M. *et al.* (2003) Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc. Natl. Acad. Sci. U. S. A* 100, 3455-3460

11 Bonen,D.K. *et al.* (2003) Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology* 124, 140-146

12 Rogler,G. (2004) Update in inflammatory bowel disease pathogenesis. *Curr. Opin. Gastroenterol.* 20, 311-317

13 Hisamatsu,T. *et al.* (2003) CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 124, 993-1000

14 Kobayashi,K.S. *et al.* (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307, 731-734

15 Ogura,Y. *et al.* (2003) Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 52, 1591-1597

16 Wehkamp,J. *et al.* (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53, 1658-1664

17 Netea,M.G. *et al.* (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052-2059

18 Watanabe,T. *et al.* (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* 5, 800-808

19 Maeda,S. *et al.* (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307, 734-738

20 Li,J. *et al.* (2004) Regulation of IL-8 and IL-1beta expression in Crohn's disease associated NOD2/CARD15 mutations. *Hum. Mol. Genet.* 13, 1715-1725

21 Travassos,L.H. *et al.* (2004) Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* 5, 1000-1006

22 Traub,S. *et al.* (2004) Structural requirements of synthetic muropeptides to synergize with lipopolysaccharide in cytokine induction. *J. Biol. Chem.* 279, 8694-8700

23 van Heel,D.A. *et al.* (2005) Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur. J. Immunol.* 35, 2471-2476

24 Fritz,J.H. *et al.* (2005) Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and. *Eur. J. Immunol.* 35, 2459-2470

25 Girardin,S.E. and Philpott,D.J. (2004) Mini-review: the role of peptidoglycan recognition in innate immunity. *Eur. J. Immunol.* 34, 1777-1782

26 Netea,M.G. *et al.* (2005) Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J. Immunol.* 174, 6518-6523

27 Mengin-Lecreulx,D. *et al.* (1999) Expression of the Staphylococcus aureus UDP-N-acetylmuramoyl-L-alanyl-D-glutamate:L-lysine ligase in Escherichia coli and effects on peptidoglycan biosynthesis and cell growth. *J. Bacteriol.* 181, 5909-5914

28 Mengin-Lecreulx,D. and van,H.J. (1985) Effect of growth conditions on peptidoglycan content and cytoplasmic steps of its biosynthesis in Escherichia coli. *J. Bacteriol.* 163, 208-212

29 Kang,D. *et al.* (1998) A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc. Natl. Acad. Sci. U. S. A* 95, 10078-10082

30 Liu,C. *et al.* (2000) Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. *J. Biol. Chem.* 275, 24490-24499

31 Liu,C. *et al.* (2001) Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J. Biol. Chem.* 276, 34686-34694

32 Girardin,S.E. *et al.* (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep.* 2, 736-742

33 Ogura,Y. *et al.* (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J. Biol. Chem.* 276, 4812-4818

34 Bertin,J. *et al.* (1999) Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J. Biol. Chem.* 274, 12955-12958

35 Inohara,N. *et al.* (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J. Biol. Chem.* 274, 14560-14567

36 Chin,A.I. *et al.* (2002) Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* 416, 190-194

37 Kobayashi,K. *et al.* (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416, 194-199

38 Vavricka,S.R. *et al.* (2004) hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. *Gastroenterology* 127, 1401-1409

39 Mellroth,P. *et al.* (2005) Ligand-induced dimerization of Drosophila peptidoglycan recognition proteins in vitro. *Proc. Natl. Acad. Sci. U. S. A* 102, 6455-6460

40 Philpott,D.J. *et al.* (2001) Innate immune responses of epithelial cells following infection with bacterial pathogens. *Curr. Opin. Immunol.* 13, 410-416

41 McGovern,D.P. *et al.* (2005) Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. *Hum. Mol. Genet.* 14, 1245-1250

42 Flouret,B. *et al.* (1981) Reverse-phase high-pressure liquid chromatography of uridine diphosphate N-acetylmuramyl peptide precursors of bacterial cell wall peptidoglycan. *Anal. Biochem.* 114, 59-63

43 Stenbak,C.R. *et al.* (2004) Peptidoglycan molecular requirements allowing detection by the Drosophila immune deficiency pathway. *J. Immunol.* 173, 7339-7348

44 Inohara,N. *et al.* (2001) Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J. Biol. Chem.* 276, 2551-2554

Chapter 13

Summary and general discussion

Summary

Innate immunity is considered our first line of defence against invading pathogens. With the discovery of pattern recognition receptors (PRRs) capable of sensing preserved molecular motifs of microbes, a giant step was made in understanding our inherited host defence mechanisms. Moreover, an important advance was achieved by developing a conceptual model to study and understand innate immune responses. The molecular mechanisms of interaction between specific ligands and PRRs were deciphered in a relative short period of time, meanwhile the picture emerged that the resulting immunological effects are very complex. This complexity is brought by the interaction between different PRRs when stimulated at the same time, as it happens during an infection, since microbes contain more than one type of ligand.

In the first part of this thesis, we have studied the interaction of two major classes of PRRs: the Toll-like receptors (TLRs) and the C-type lectin like receptors (CLRs). By simultaneous stimulation with specific ligands of TLR2, TLR4 and dectin-1, we show in **chapter 2** that in both human monocytes and macrophages this leads to synergistic TNF and IL-10 production. These data indicate that TLR and CLR signaling pathways converge to increase gene transcription, leading to enhanced production of cytokines. Almost all Toll-like receptors (with the exception of TLR3) have a common pathway starting with the adaptor molecule Myd88, leading to a cascade of interacting signal molecules and eventually achieving the activation and translocation of the transcription factor NF-κB. There are two signal pathways activated by dectin-1: a Syk-dependent and a Syk-independent route, which has recently been shown to signal through Raf-1 [1]**.** Phosphorylation of Syk leads to the translocation of NF-κB, leading to the production of proinflammatory cytokines. In contrast, binding and phagocytosis of β-glucan-containing particles is Syk-independent. In **chapter 3**, we used both human and murine models to show that the synergistic production of TNF after stimulation of both TLRs and dectin-1 is both MyD88 and Syk-dependent. This signal integration is achieved at the transcriptional level, as shown by a synergistic and sustained translocation of NF-κB after stimulation with both TLR ligands and β-glucan. Stimulation of cells with β-glucan alone leads to translocation of NF-κB, but no excretion of TNF by murine macrophages. Co-stimulation of the TLR MyD88-dependent pathway is needed to translate and excrete TNF efficiently. These results have implications for the conceptual role of PRRs in infections, because it indicates the importance of collaboration between different classes of PRR for the induction of an optimal cytokine production. While the cytokine production in dectin-1-deficient mice is not impaired after TLR stimulation alone, it is seriously impaired after stimulation with complex fungal particles activating both dectin-1 and TLR signal pathways [2].

Engagement of dectin-1 by β-glucan induces different responses in macrophages. In chapter 2 and 3 we have shown the interaction between TLRs and dectin-1 for the induction of cytokines. In **chapter 4** we further explored the dectin-1-dependent modulation of

membrane-bound molecules by β-glucan. Coordination of the immune response depends on the secretion of cytokines and direct cell-cell interaction. The up-and-down regulation of molecules expressed on the cell membrane will induce a specific response of the interacting cells through activating or inhibiting signal pathways at the level of the cell membranes. When murine macrophages are exposed to zymosan, they will phagocytose these particles and secrete cytokines. Using FACS analysis, we showed a marked pattern of regulation of membrane bound molecules after exposure to zymosan, a β-glucan-rich particle obtained from the cell wall of *Saccharomyces cerevisiae*. When we compared the expression patterns of membrane-bound molecules after exposure of cells isolated from dectin-1-deficient and wild-type mice to zymosan, several dectin-1dependently regulated molecules were identified, among which CD47. The downregulation of CD47 is directly regulated by dectin-1 signal pathways, and partly dependent on the dectin-1-mediated phagocytosis of the particles. CD47 is known to play a role in the induction of phagocytosis of apoptotic and infected cells when its expression is downregulated. We therefore propose a role for CD47 in regulating phagocytosis of infected cells via cell-cell interaction, and this might have a role in the exchange of antigens for the induction of adaptive immune responses. These findings further underline the concept of complex interaction of various activated signal pathways induced by single PRR engagement, aimed to induce effective immune responses against pathogens.

The interaction between dectin-1 and TLRs leads to enhanced cytokine production in vitro. Dectin-1 deficient mice have a greater susceptibility to fungal infections. What is the consequence when dectin-1 is not functioning in humans? If the synergistic production of cytokines is important in fungal infection and this is dectin-1-dependent, one would expect a greater susceptibility to fungal infection in humans. However, an 'Achilles heel' in immunology is rare, and usually, many redundant mechanisms are present. In **chapter 5** we describe a patient with recurrent vulvovaginal candidiasis, onychomycosis and an impaired cytokine production to fungal stimuli. Sequencing of the dectin-1 gene led to the identification of an early stop codon (Tyr238Stop mutation) in the gene encoding the carbohydrate recognition domain (CRD), which is responsible for β-glucan recognition. Further analysis of the family revealed that both parents were heterozygous and two siblings were homozygous for this mutation. Both siblings and their mother also suffered from chronic onychomycosis. The mutated form of dectin-1 was poorly expressed and functional analysis on the cellular level showed that the synergism between TLRs and dectin-1was impaired. Consequently, cytokine production induced by *C. albicans* was lower compared to healthy controls, and the binding of *C. albicans* was impaired. However, the phagocytosis and killing of *C. albicans* was unaffected. These results help us to define the role of dectin-1 within the framework of anti-fungal defense mechanisms, pointing at an important role of dectin-1 in mucosal defense. In the case of invasive candidiasis, the fungicidal capacity of phagocytes like neutrophils are crucial, which seems to be unaffected by dectin-1 defects. Knowing the functional consequences of this mutation and the phenotype, we subsequently studied the global occurrence of the polymorphism in different ethnic populations, and we attempted to identify when it first emerged. We used a phylogenetic-based approach to

show that this mutation lies in an evolutionary conserved area of the mammalian lineage. In further analysis of the prevalence of this mutation in populations with different ethnic backgrounds representing the populations of the major continental masses: Dutch Caucasians (Europe), Tanzanians (Africa), Han Chinese (Asia) and Surinam (Native Indians), we show that this mutation is only present in the European population (allele frequency 0.069) and in the African population (0.035). This points at an ancient origin of this mutation, which was confirmed by haplotype analysis of two large cohorts of European and African populations. This analysis also revealed that there is a relative high prevalence of the mutation in European and African populations. It is known that the prevalence of mucosal candidiasis is high (5% of all women have RVVC) and this disease most probably has a multifactorial cause, of which this dectin-1 polymorphism may represent an important genetic factor.

As we have shown that there is an interaction between dectin-1 and TLRs, which in turn seems to play an important role in the antifungal mucosal immunity, this could have implications for the development of new therapeutic strategies. In **chapter 6** we reviewed the literature on the pattern recognition of *C. albicans* and we have placed these findings into the perspective of vaccine development. The clearest conclusion one can draw regards the importance of the innate immune system in the host defense against *C. albicans*. First of all, the cytokines induced after recognition of a fungal pathogen by the innate immune system are crucial for the activation and coordination of the cellular and humoral response. The TLRs are strong inducers of inflammatory responses, while the CLRs are potent modulators of these responses by enhancing or inhibiting cytokine production. Secondly, the proper cocktail of cytokines should be induced for an optimal protection against mucosal candidiasis. This is illustrated by patients with Hyper IgE syndrome, who have mutations in STAT3, leading to an impaired IL-17 production and consequently to an impaired Th17 response [3]. These patients suffer from severe mucosal candidiasis. Thirdly, it seems that the induction of the cellular immune response, especially the induction of Th1 and Th17, leads to an optimal protection against *C. albicans* infection at mucosal sites. In general, it is concluded that for the development of potent vaccines against *C. albicans*, a combination of both CLR and TLR ligands should be used as adjuvants for the induction of specific cellular immune responses against *C. albicans*.

In the second part of this thesis we have assessed the interaction between Toll-like receptors and the Nod-like receptor Nod2. Nod2 is an intracellular receptor of bacterial peptidoglycans that is a member of the NLR family of pattern recognition receptors. A defective recognition of peptidoglycans by mutated forms of Nod2 has been linked to a strongly increased susceptibility to Crohn's disease. In order to investigate the interaction of Nod2 with other classes of PRRs, we have used cells of patients with Crohn's disease homozygous for the 3020insC mutation, resulting in a defective recognition domain for muramyl dipeptide (MDP). By using pure ligands for TLRs and Nod2, we show in **chapter 7** that simultaneous stimulation of both classes of receptors leads to a synergistic production of cytokines by PBMC, which was abolished in the cells bearing the 3020insC mutation. Both TLR2 and Nod2

are important for the induction of cytokines by peptidoglycan (PGN), and this interaction is impaired in cells from patients with the 3020insC mutation, leading to a defective TNF and IL-10 production. This impaired production of the anti-inflammatory cytokine IL-10 could lead to a bias to a Th1 response, potentially playing a role in the inflammation in Crohn's disease (Fig 1).

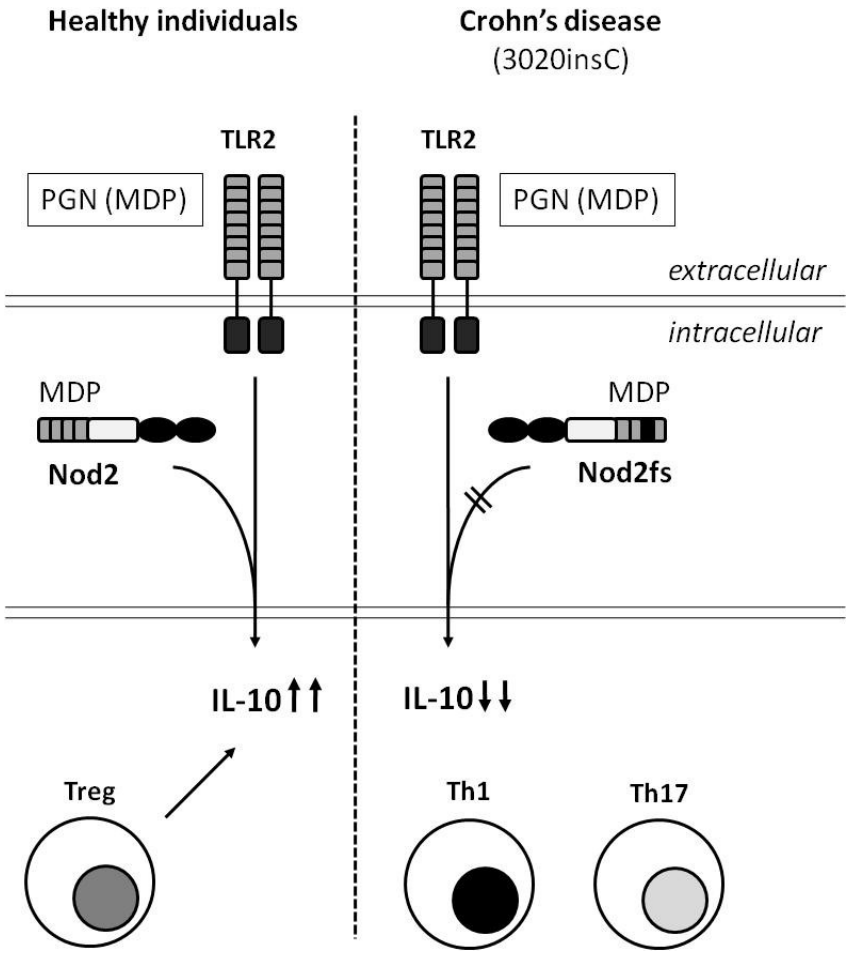


Figure 1. Impaired IL-10 production leads to pro-inflammatory T*helper* cells. Activation of the inflammatory signaling pathways by peptidoglycan (PGN) is mediated by TLR2 and Nod2. Engagement of both receptors will lead to a synergistic production of cytokines such as IL-10 and differentiation of T-cells into the anti-inflammatory T*regulatory* (Treg) cells. The recognition of MDP by Nod2 is impaired in Crohn's disease patients with the 3020insC mutation, and the activated signaling pathway of TLR2 is not amplified, leading to a relative low production of IL-10. This will lead to an impaired differentiation of T-cells into Treg cells, and subsequent to a bias towards the proinflammatory Th1 and Th17 cells.

The synergy between TLRs and Nod2 was also demonstrated for the induction of IL-1β, a potent pro-inflammatory cytokine. This cytokine is produced as a pro-peptide (proIL-1β) which has to be cleaved by caspase-1 into the bioactive form (IL-1β), which is subsequently secreted to exert its role in inflammatory responses. In **chapter 8** we further investigate the role of Nod2 for the induction of IL-1β, and show that Nod2 is crucial for induction of transcription of proIL-1β and the secretion of IL-1β. The synergy between TLRs and Nod2 for

the induction of IL-1β is mediated at the post-translational level in a caspase-1-dependent manner. The exact mechanism for the activation of caspase-1 by Nod2 has yet to be clarified, although it has been shown that other members of the NLR family such as NALP3 form protein complexes named inflammasomes, which activate caspase-1. An interaction has been demonstrated between Nod2 and NALP1, another member of NLRs recently shown to form an active inflammasome [4]. Interestingly, the synergy in TNF production after stimulation with MDP and LPS (a TLR4 ligand) is induced at transcriptional level. This shows that the cross-talk between TLRs and Nod2 for the induction of cytokines is exerted at different levels, depending on the cytokine induced.

MDP-containing peptidoglycans are an important component of the cell wall of Gram-positive bacteria, including mycobacteria. In the next two chapters, we investigated the role of Nod2 and its crosstalk with TLR in the inflammatory response against mycobacteria. In **chapter 9** we show that *Mycobacterium tuberculosis* is recognized by Nod2 and induces production of cytokines in human PBMC in a Nod2- dependent manner. By using purified cell wall components of *M. tuberculosis*, we show that there is a synergistic cytokine production after stimulation with the TLR2 ligand 19kDa lipoprotein and MDP, and this synergism was lost in cells bearing the 3020insC mutation. Because the non-redundancy of this pathway, and the crosstalk between both TLR and Nod2 recognition systems, the cytokine induction by *M. tuberculosis* was impaired in cells of Crohn's disease patients bearing the 3020insC mutation. Crohn's disease has thought to be associated with *M. paratuberculosis* for many years, although its role in the pathogenesis is controversial. In **chapter 10** we show that *M. paratuberculosis* is also recognized by TLR2, TLR4 and Nod2 (Fig 2). The cytokine production by cells of Crohn's disease patients bearing the 3020insC mutation after stimulation with *M. paratuberculosis* was impaired. Although speculative, these results are compatible with the idea that an impaired immune response against intracellular pathogens like *M. paratuberculosis* may play a role in the pathogenesis of Crohn's disease.

As shown in the previous chapters, a synergistic crosstalk exists between TLRs and Nod2, leading to an enhanced cytokine production by primary human mononuclear cells when stimulated simultaneously. This resembles the simultaneous activation of these pathways during infections. However, this differs from the situation in the gut where there is a continuous exposure to commensal microorganisms and their fragments. Indeed, MDP can be found passing the mucosal barrier in gut tissue. In **chapter 11** we preincubated PBMC with MDP to mimic the chronic exposure to bacterial fragments, and subsequently stimulated these cells with TLR ligands and intestinal pathogens. With these experiments we show that continuous exposure to the Nod2 ligand MDP leads to a decreased TLR4-induced TNF production, whereas the TLR2 pathway was unaffected. This cross tolerance to TLR4 ligands might be an important mechanism to explain how Nod2 mutations can lead to chronic inflammation of the gut, as seen in Crohn's disease. In 3020insC cells, this cross tolerance was abolished, leading to a sustained TNF production, a cytokine central in the pathogenesis of Crohn's disease (Fig 3).

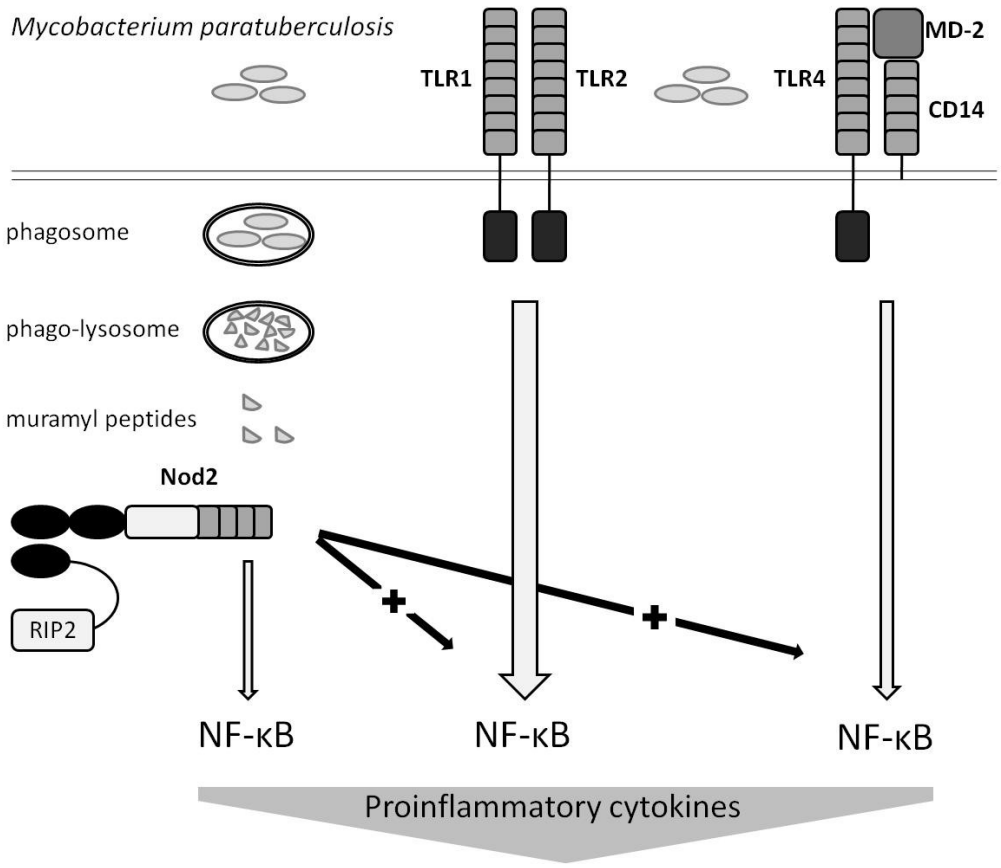


Figure 2. *Mycobacterium paratuberculosis* activates both TLR and Nod2 signaling pathways. Stimulation of human mononuclear cells with *M. paratuberculosis* leads to secretion of proinflammatory cytokines. M. paratuberculosis is a strong inducer of cytokine production through the TLR2 pathway, and a weaker activator of the TLR4 signal pathway. M. *paratuberculosis* also activates the Nod2 signal pathway leading to a synergistic interaction with the TLR signal pathway resulting in an enhanced proinflammatory cytokine production.

This model for the pathogenesis of Crohn's disease is strengthened by the association of mutations in the TLR4 pathways leading to a higher incidence in different populations. The mechanism through which Nod2 plays a role in the pathogenesis of Crohn's disease is still controversial: how can a loss of the potential for recognition of MDP, the ligand for Nod2, lead to a pro-inflammatory disease? The loss of cross tolerance between Nod2 and TLRs, leading to an enhanced production of pro-inflammatory cytokines, might be one explanation. In addition, the decreased production of the anti-inflammatory cytokine IL-10 and the subsequent bias towards Th1 responses is also proposed to play a role in the gut inflammation. In both models, the interaction between different classes of PRRs is crucial, and this underlines the immunomodulatory capacity of MDP through Nod2 signaling.

In **chapter 12** we investigated the immunomodulatory effects of other muramyl peptides that are known to be ligands of Nod1, another important NLR receptor. The minimal motif to stimulate Nod1 is muramyl tripeptide (MTP), a component of the peptidoglycan layer of Gram-negative bacteria. In parallel with MDP, stimulation of PBMC with MTP and TLR ligands

simultaneously leads to a synergistic production of cytokines. Interestingly, cells of patients bearing the 3020insC mutation were unresponsive to stimulation with MTP, suggesting a common signal pathway for both Nod1 and Nod2, which is affected by this mutation. However, in these cells the synergism between Nod1 and TLRs was unaffected, indicating a different signal pathway involved in the cross-talk between TLRs and Nod1, then the direct induction of cytokines by Nod1 and Nod2. Different studies have shown that indeed the members of the NLR family exert their function at different levels: 1. direct NF-κB activation resulting in transcription of cytokines; 2. forming complexes with other molecules to activate enzymes and modulate post-translational processes; 3. mediating ATP signaling for the secretion of cytokines; and 4. the regulation of transcriptional factors independent of MDP activation [5]. Al these findings underline the potential role of the NLR family in modulating immune responses through complex interaction with many signal pathways induced by TLR stimulation.

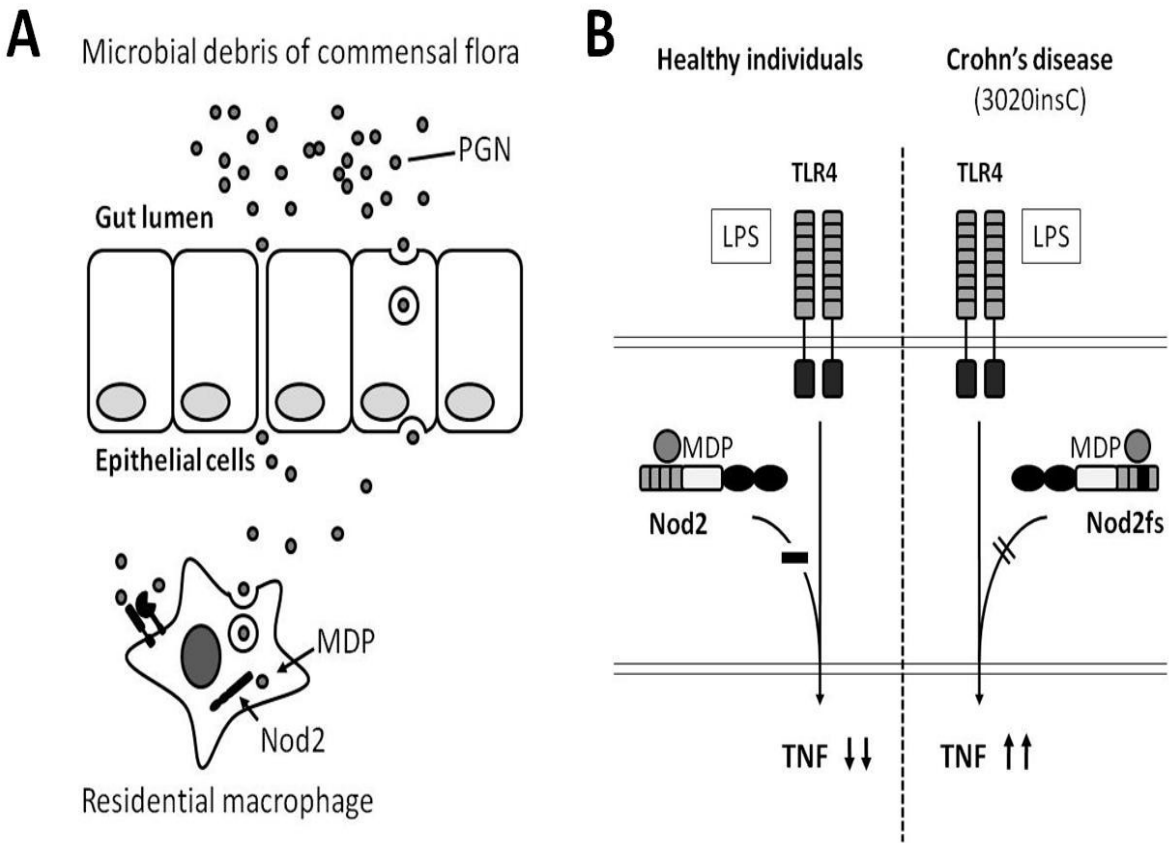


Figure 3. Continuous exposure to MDP leads to inhibition of TLR4-induced TNF production. A: The luminal side of the gut is continuously exposed to microbial compounds, like peptidoglycan (PGN), of the commensal microbial flora. PGN can pass the epithelial barrier and can be phagocytosed by residential macrophages, leading to chronic stimulation of Nod2. B: in healthy individuals continuous stimulation of Nod2 with MDP leads to inhibition of TLR4-induced TNF production by lipopolysacharide (LPS), whereas in cells homozygous for the 3020insC mutation this inhibition is impaired, resulting in a higher TNF production after stimulation with LPS. This mechanism might explain how the 3020insC mutation leads to a sustained TNF production and chronic inflammation.

General conclusion

In this thesis, I have demonstrated the presence of complex cross-talk networks between different classes of PRRs, leading to enhancement or inhibition of cytokine responses induced by microorganisms or their ligands. These immunomodulatory effects play a role for host defense to infections (resistance), but also for the homeostasis between host and commensal flora (tolerance). When the interaction between PRRs is disrupted (by mutations, inhibition by pathogens, or medication) this could lead to disease in two ways. First of all, an impaired immune response against pathogens caused by insufficient production of cytokines can lead to a greater susceptibility to pathogens. Secondly, an impaired tolerance caused by insufficient downregulation of pro-inflammatory cytokines can lead to chronic inflammation caused by commensal flora. Insight into these mechanisms will be very important for the development of new therapeutic strategies. For induction of greater resistance against pathogens through vaccination, enhancement of cytokines leading to the proper cellular responses, likeTh1 and Th17, will be crucial. The development of new adjuvants based on synergy between several PRR signal pathways can be used to achieve this. Illustrative is the use of CpG, a TLR 9 agonist, as an adjuvant to boost Th1 responses. For the induction of increased tolerance, downregulation of inflammatory signaling pathways induced by commensal flora may be desirable, leading to relative enhancement of anti-inflammatory cellular responses, like induction of Th2 and Treg. Part of the beneficial effect of probiotics, and certain nutritional supplements might be ascribed to this mechanism. Insight into the induction of tolerance through PRR stimulation has received much attention in medical research and might lead to the development of new therapies in chronic inflammatory diseases like Crohn's disease.
In conclusion, the innate immune responses are complex processes in which the interaction between various classes of PRRs plays a central role. In this thesis, we attempted to shed light on several aspects of this complex network, in order to provide the basic knowledge for new potential therapies in infections and autoinflammatory diseases.

Reference List

1 Gringhuis,S.I. *et al.* (2009) Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat. Immunol.* 10, 203-213

2 Taylor,P.R. *et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31-38

3 Ma,C.S. *et al.* (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J. Exp. Med.* 205, 1551-1557

4 Hsu,L.C. *et al.* (2008) A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proc. Natl. Acad. Sci. U. S. A* 105, 7803-7808

5 Noguchi,E. *et al.* (2009) A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1. *Nat. Immunol.* 10, 471-479

Nederlands

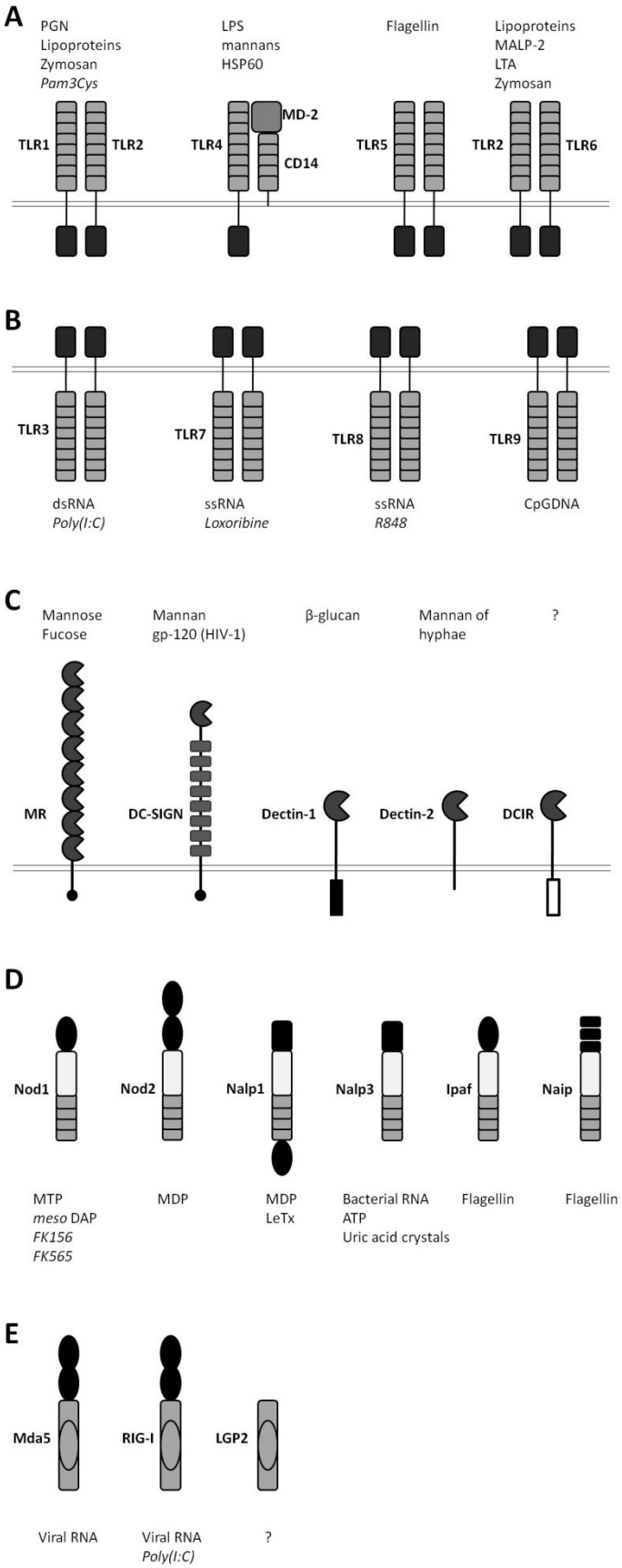
Samenvatting en algemene discussie

Samenvatting en algemene discussie

De interactie tussen gastheer en micro-organismen fascineert wetenschappers al vele eeuwen en hun onderzoek heeft geleid tot de ontdekking van vaccinatie en antibiotica, die beiden een grote rol hebben gespeeld in de geschiedenis van de mensheid. Onze afweer wordt ingedeeld in de aangeboren afweer en de verworven afweer. De aangeboren afweer wordt gezien als eerstelijns verdediging tegen ziekteverwekkers, hierin spelen fagocyterende cellen een centrale rol. Hoe deze fagocyten micro-organismen kunnen herkennen is heel lang een raadsel geweest.

Sinds de ontdekking van patroon-herkennende immuunreceptoren (in het Engels worden deze *pattern recognition receptors* genoemd en afgekort met **PRRs**) die moleculaire patronen kunnen waarnemen die specifiek zijn voor micro-organismen (*pathogen associated molecular pattern*, afgekort als **PAMP**), is er een enorme vooruitgang geboekt in het begrijpen van dit aangeboren afweersysteem. Daarnaast is het belangrijk geweest dat er een conceptueel model van deze aangeboren immuunrespons ontwikkeld is, waardoor het bestuderen van deze respons snel tot meer inzicht heeft geleid. De moleculaire interactie tussen specifieke microbiële structuren (liganden) en PRRs is in een relatief korte tijd ontrafeld, waar bij er meerdere klasse PRRs zijn ontdekt (Fig 1). Binding van liganden aan PRRs leidt tot een intracellulaire cascade van interacterende eiwitten die we 'signal pathway' noemen. Een *pathway* is een reeks van eiwitten waarbij het ene eiwit telkens het volgende eiwit aan- of uitschakelt (stimuleert of inhibeert). Inzicht in deze *signal pathways* en de secretie van eiwitten die immuun cellen kunnen aansturen (cytokines) door fagocyten maakte duidelijk dat de aangeboren afweer ook een belangrijke rol speelt in de modulatie van de verworven immuunrespons (Fig 2). Een belangrijke vraag bleef echter, hoe relatief weinig receptoren in staat zijn om een specifieke immuunrespons te induceren tegen onvoorstelbaar veel verschillende pathogenen. Een deel van deze specificiteit zou kunnen worden verklaard door de interactie tussen de verschillende PRRs wanneer deze gelijktijdig worden gestimuleerd, zoals dit gebeurt tijdens infecties omdat micro-organismen meerdere liganden bevatten.

Figuur 1. Patroon-herkennende immuunreceptoren en hun liganden. Er zijn vier verschillende klassen patroon herkennende receptoren bekend: **Toll-like receptoren** die tot expressie komen op de celmembraan (A) en in het fagosoom (B) hebben een herkenningsdomein met *leucine rich repeats* (LRR) en een TLR/IL-1 receptor (TIR) domein; **C-type lectin receptoren** die op de celmembraan en in het fagosoom tot expressie komen (C) hebben een carbohydraat herkenningsdomein; In het cytoplasma komen **Nod-like receptoren** voor die een nucleotide oligomerisatie domein en meestal een LLR domein bevatten (D); en **RigI helicases** met een helicase domein een C-terminaal repressie domein (E).


A
PGN
Lipoproteins
Zymosan
Pam3Cys
LPS
mannans
HSP60
Flagellin
Lipoproteins
MALP-2
LTA
Zymosan
TLR1
TLR2
TLR4
MD-2
CD14
TLR5
TLR2
TLR6
B
TLR3
TLR7
TLR8
TLR9
dsRNA
Poly(I:C)
ssRNA
Loxoribine
ssRNA
R848
CpGDNA
C
Mannose
Fucose
Mannan
gp-120 (HIV-1)
β-glucan
Mannan of
hyphae
?
MR
DC-SIGN
Dectin-1
Dectin-2
DCIR
D
Nod1
Nod2
Nalp1
Nalp3
Ipaf
Naip
MTP
meso DAP
FK156
FK565
MDP
MDP
LeTx
Bacterial RNA
ATP
Uric acid crystals
Flagellin
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RIG-I
LGP2
Viral RNA
Viral RNA
Poly(I:C)
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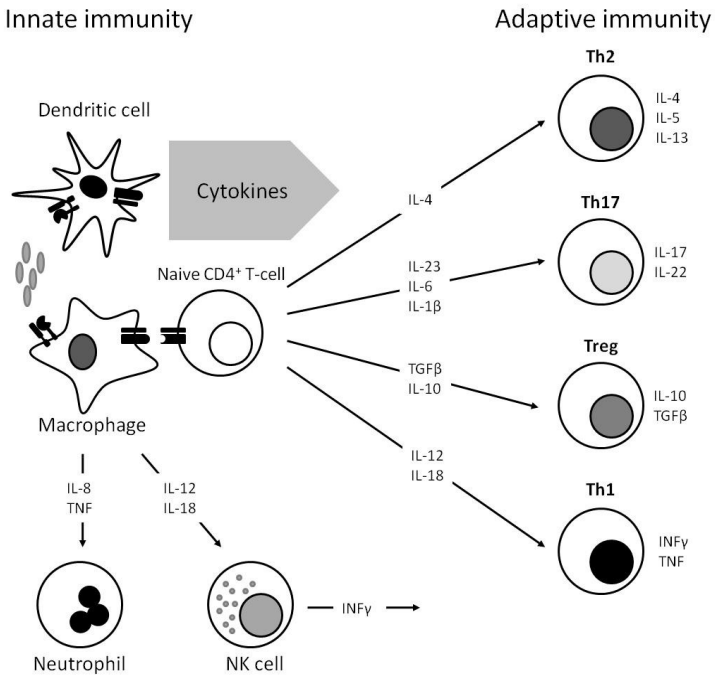

Figuur 2. Patroon-herkennende immuunreceptoren (PPRs) reguleren de adaptieve immuunrespons. Dendritische cellen en macrofagen (antigeen presenterende cellen (APC)), neutrofielen en natural killer cellen zijn de belangrijkste cellen van de aangeboren immuuncellen, terwijl de adaptieve immuun cellen bestaan uit B-cellen en T-cellen. De PRRs komen tot expressie op de APCs. Als een microbieel ligand aan een PRR bindt, wordt er een *signal pathway* geactiveerd en cytokines geproduceerd. Na dat het micro-organisme is gefagocyteerd en afgebroken in het fagolysosoom, worden antigenen gepresenteerd op het MHCII molecuul. Tijdens dit proces worden ook intracellulaire PRRs geactiveerd. Expressie van co-stimulerende moleculen op de antigeen-presenterende cel worden door activatie van PRR verhoogd. Het antigeen/MHCII complex en de co-stimulerende moleculen op de APC activeren de T-celreceptor en induceert proliferatie van de naïeve T-cel. De cytokines die door de APC worden geproduceerd zijn cruciaal voor de differentiatie van de T-cel in verschillende T*helper*-cellen.

In het eerste deel van dit proefschrift hebben we de interactie tussen twee belangrijke groepen van PRRs bestudeerd: de Toll-like receptoren (TLRs) en de C-type lectin like receptoren (CLRs). Door gelijktijdig stimulaties van TLR2, TLR4 en dectin-1 met specifieke liganden laten we in **hoofdstuk 2** zien dat dit leidt tot een synergisme in TNF en IL-10 productie in humane monocyten en macrofagen. Dit duidt er op dat TLR en CLR *signal pathways* convergeren en leiden tot verhoogde transcriptie van genen en een toegenomen cytokineproductie. Vrijwel alle Toll-like receptoren (met uitzondering van TLR3) hebben een gemeenschappelijke *pathway* die start bij adapter molecuul MyD88 en na een cascade van interacterende signaal moleculen leidt tot de activatie en translocatie van de transcriptie-factor NF-κB. Er worden twee *signal pathways* geactiveerd door dectin-1: een Syk-afhankelijke en een Syk-onafhankelijke route, waarvan recent is aangetoond dat deze via Raf-1 loopt. Fosforilatie van Syk leidt tot translocatie van NF-κB en de productie van pro-inflammatoire cytokines. Dit in tegenstelling tot activatie van de Syk-onafhankelijke route die leidt tot binding en fagocytose van β-glucan-bevattende deeltjes. In **hoofdstuk 3** hebben we in zowel muizen als humane cellen laten zien dat de synergistische productie van TNF na

stimulatie van TLRs en dectin-1 zowel MyD88 als Syk afhankelijk is. Beide signalen worden geïntegreerd op transcriptie-niveau, zoals we laten zien door een toegenomen en aanhoudende translocatie van NF-κB na stimulatie met TLR liganden en β-glucan. Stimulatie met alleen β-glucan leidt wel tot translocatie van NF-κB, maar niet tot excretie van TNF door macrofagen. Co-stimulatie van de TLR MyD88 afhankelijke *pathway* is nodig voor translatie en secretie van TNF. Deze resultaten hebben invloed op de conceptuele rol die PRRs hebben tijdens infecties, omdat het belang van samenwerking tussen de verschillende PRRs wordt aangetoond voor een optimale cytokine productie. In dectin-1-deficiënte muizen is de cytokine productie na stimulatie van alleen TLRs niet verminderd, echter na stimulatie met complexe fungale deeltjes die zowel TLRs als dectin-1 stimuleren is de cytokineproductie ernstig gestoord.

Stimulatie van dectin-1 induceert verschillende responsen in macrofagen. In hoofdstuk 2 en 3 hebben we de interactie tussen TLRs en dectin-1 voor de inductie van cytokines laten zien, in **hoofdstuk 4** hebben we onderzocht of dectin-1 betrokken is bij de modulatie van membraangebonden moleculen door β-glucan. De coördinatie van de immuunrespons wordt geregeld door de secretie van cytokines en directe cel-cel interactie. De up-and-down-regulatie van moleculen die tot expressie komen op het celmembraan van fagocyten induceren een specifieke respons van de interacterende cel door activatie of inhibitie van *signal pathways* op het niveau van het celmembraan. Muizen macrofagen fagocyteren zymosan, dit bestaat uit de wand van *Saccharomyces cerevisiae* en bevat veel β-glucan, en secreteren dan cytokines. Uit FACS analyse blijkt dat er een specifiek patroon van membraan gebonden eiwitten ontstaat na stimulatie met zymosan. Door het vergelijken van de expressie patronen van dectin-1-deficiënte macrofagen met controle macrofagen, konden verschillende dectin-1 afhankelijk gereguleerde moleculen worden geïdentificeerd, waaronder CD47. Deze verminderde expressie van CD47 is direct gereguleerd door dectin-1 *signal pathways* en deels door fagocytose van partikels. CD47 induceert fagocytose van apoptotische en geïnfecteerde cellen wanneer de expressie op het celmembraan is afgenomen. Mogelijk speelt de verminderde expressie van CD47 door dectin-1-binding een rol in de regulatie van fagocytose van geïnfecteerde cellen via cel-cel interactie. Hierdoor is uitwisseling van antigenen mogelijk voor een optimale adaptieve immuunrespons. Deze bevindingen benadrukken de complexe interactie van verschillen geactiveerde pathways door binding van een enkele PRR in de inductie van een effectieve immuunrespons tegen pathogenen.

De interactie tussen dectin-1 en TLRs induceert een toegenomen cytokine productie van fagocyten in vitro en dectin-1 deficiënte muizen zijn gevoeliger voor schimmel infecties. Wat is de consequentie als dectine-1 niet functioneert in mensen? Als het synergisme in cytokineproductie belangrijk is in de afweer tegen schimmels en deze dectin-1 afhankelijk is, dan zou er ook voor mensen een verhoogde gevoeligheid voor schimmel infecties in zijn. Hier tegenover staat dat een ‘Achilles hiel’ in de immunologie zeldzaam is en dat er vaak meerdere compensatie mechanismen aanwezig zijn. In **hoofdstuk 5** presenteren we een patiënt met een recidiverende vaginale *Candid*- infecties en onychomycosis (schimmel

nagels) met een sterk verminderde cytokine door fagocyten na stimulatie met schimmels. Bepaling van de nucleïnezuursequentie van het dectin-1-gen toonde aan dat er sprake was van een mutatie die resulteerde in een stop-codon (Tyr238Stop) in het gedeelte dat codeert voor het herkenningsdomein van dectin-1 voor β-glucan. Verder analyse van de familie liet zien dat beide ouders heterozygoot waren en twee zussen homozygoot voor dezelfde mutatie. Beide zussen en de moeder hadden ook onychomycosis en vaginale schimmelinfecties. Dectin-1 met deze mutatie kwam nauwelijks tot expressie en functionele analyse op cellulair niveau liet een sterk afgenomen synergisme in cytokineproductie zien tussen TLRs en dectin-1. Als gevolg hiervan was ook de cytokineproductie na stimulatie met *C. albicans* afgenomen in vergelijking et gezonde controle personen, daarnaast was ook de binding aan fagocyten afgenomen. De fagocytose en killing van *C. albicans* was echter niet veranderd. Deze resultaten helpen ons de rol van dectin-1 te definiëren binnen het framewerk van afweermechanismen tegen schimmels, namelijk dat dectin-1 belangrijk is in de mucosale immuniteit. In de afweer tegen invasieve schimmels spelen fagocytose door neutrofielen een cruciale rol, deze was echter niet veranderd door de dectin-1-mutatie. Na het vaststellen van de functionele consequenties en het fenotype van deze mutatie hebben we vervolgens het voorkomen in verschillend etnische populaties wereldwijd bestudeerd, om te achterhalen wanneer deze mutatie voor het eerst voorkwam. Uit een fylogenetische benadering blijkt dat deze mutatie in een evolutionair geconserveerd gebied ligt van de zoogdieren-lijn. In de verdere analyse naar het voorkomen van deze mutatie in populaties met een verschillende etnische achtergrond die de belangrijkste continenten representeren: Nederlands caucasische (Europa), Tanzaniaanse (Afrika), Han Chinese (Azië) en Surinaamse (Inheemse Amerikanen), laten we zien dat deze mutatie alleen aanwezig is in de Europese populatie (allel-frequentie 0.069) en in de Afrikaanse populatie (allel-frequentie 0.035). Dit duidt erop dat de mutatie al zeer oud is. Door middel van haplotypering in twee grote cohorten uit Europa en Afrika is dit bevestigd. Uit deze analyse blijkt ook dat deze mutatie vrij veel in de populatie voorkomt in Afrika en Europa. Het is dan ook bekend dat mucosale *Candida* vaak voorkomen (5% van de vrouwen heeft recidiverende vaginale *Candida*-infecties) en dat er meest waarschijnlijk een multifactoriële oorzaak aan ten grondslag ligt, waarvan deze dectin-1-polymorfisme een belangrijke genetische factor kan zijn.

Uit het voorgaande blijkt dat er interactie bestaat tussen dectin-1 en TLRs, en dat deze interactie een belangrijke rol speelt in de afweer tegen mucosale schimmels, maar kan dit ook gebruikt worden voor nieuwe therapieën? In hoofdstuk 6 wordt er een overzicht gegeven van de literatuur die over PRRs en de herkenning van *C. albicans* gaat en wordt belicht hoe deze kennis gebruikt kan worden voor vaccin-ontwikkeling. Het meest duidelijk komt er naar voren dat het aangeboren afweersysteem een cruciale rol speelt bij de afweer tegen *C. albicans*. Ten eerste is de cytokineproductie die wordt geïnduceerd na herkenning van *C. albicans* door het aangeboren afweersysteem essentieel voor de activatie en coördinatie van cellulaire en humorale afweer. De TLRs induceren een sterke inflammatoire respons, terwijl de CLRs een sterk modulerend effect hebben door inhibitie of synergie van de cytokineproductie. Ten tweede blijkt de juiste combinatie van verschillende cytokines te leiden tot een optimale bescherming tegen mucosale schimmelinfecties. Dit wordt

geïllustreerd door patiënten met het hyper-IgE-syndroom, waarbij een mutatie in STAT3 leidt tot een verminderde IL-17 productie en als gevolg hiervan een verminderde Th17-respons. Deze patiënten hebben zeer ernstige mucosale schimmelinfecties. Ten derde komt naar voren dat inductie van de cellulaire Th1 en de Th17 respons cruciaal is voor optimale bescherming tegen mucosale schimmel infecties. Er wordt geconcludeerd dat adjuvants die zowel TLRs en CLRs stimuleren kunnen worden gebruikt om een specifiek cellulaire respons tegen *C. albicans* te induceren die tot bescherming tegen schimmelinfecties leidt.

In het tweede deel van dit proefschrift wordt de interactie tussen Toll-like receptoren en de Nod-like receptor Nod2 onderzocht. Nod2 is een intracellulaire receptor die bacteriële peptidoglycanen kan herkennen en behoort tot de NLR familie van patroon-herkennende receptoren. Een verhinderde herkenning van peptidoglycanen door mutaties in het Nod2-gen is sterk geassocieerd met de ziekte van Crohn. Om de interactie tussen Nod2 en TLRs te bestuderen, hebben we cellen van patiënten met de ziekte van Crohn en een homozygote 3020insC mutatie in het Nod2-gen gebruikt. Door middel van stimulatie met zuivere liganden voor TLRs en Nod2 tonen we in **hoofdstuk 7** aan dat gelijktijdige stimulatie van beide receptoren in leukocyten er een synergie in cytokine productie ontstaat. Deze synergie is afwezig in cellen met de 3020insC mutatie. Zowel TLR2 als Nod2 zijn belangrijk voor de herkenning van peptidoglycan en de inductie van cytokines, maar bij cellen van patiënten met de 3020insC mutatie is dit verstoord, waardoor er een verminderde productie van TNF en IL-10 optreedt. Deze verminderde productie van de anti-inflammatoire cytokine IL-10 kan leiden tot een bias naar een pro-infalmmatoire cellulaire Th1-respons en speelt mogelijk een rol in het ontstaan van de ziekte van Crohn.

De synergie tussen TLRs en Nod2 bestaat ook voor de inductie van de pro-inflammatoire cytokine IL-1β. Deze cytokine wordt geproduceerd als een pro-peptide (pro-IL1β) en moet eerst worden geactiveerd door caspase-1, om vervolgens te worden uitgescheiden om een biologisch effect te hebben in de inflammatoire respons. In **hoofdstuk 8** wordt de rol van Nod2 in de inductie van IL-1β verder onderzocht. Er wordt aangetoond dat Nod2 voor zowel de inductie van pro-IL1β transcriptie als de excretie van actieve IL-1β essentieel is. De synergie tussen TLRs en Nod2 voor de inductie van IL-1β ontstaat op post-translationeel niveau en is afhankelijk van caspase-1-activatie. Hoe caspase-1 door Nod2 geactiveerd wordt is nog niet bekend, wel is het bekend dat andere leden van de NLR familie, door middel van eiwit complexen die we inflammasomen noemen, betrokken zijn bij de activatie van caspase-1. Recent is aangetoond dat Nod2 een interactie kan aangaan met NALP1, een ander lid van de NLR familie waarvan bekend is dat deze een inflammasoom kan vormen. Het is interessant dat de synergie voor de productie van TNF na stimulatie van Nod2 en TLR4 op transcriptie niveau ontstaat. Dit toont aan dat de interactie van de TLR en Nod2 *signal pathways* op verschillend niveaus plaats vindt, afhankelijk van de cytokine die wordt geïnduceerd.

MDP-bevattende peptidoglycanen zijn een belangrijke component van de celwand van Gram-positieve bacteriën en ook mycobacteriën. In de volgende twee hoofdstukken hebben

we de rol van de interactie tussen TLRs en Nod2 in de aangeboren immuunrespons tegen mycobacteriën onderzocht. In **hoofdstuk 9** laten we zien dat *Mycobacterium tuberculosis* door Nod2 wordt herkend en essentieel is voor de productie van cytokines door leukocyten. Door gezuiverde componenten van de celwand van *M. tuberculosis* te gebruiken, wordt er aangetoond dat er een synergisme in cytokineproductie ontstaat na stimulatie van TLR2 met 19kDa lipoproteïne en Nod2 met MDP. Deze synergie is er niet in de cellen van Crohn-patiënten met de 3020insC mutatie. Omdat er geen alternatieve pathways zijn die tot deze synergie kunnen leiden, is de inductie van cytokines door *M. tuberculosis* ernstig gestoord bij Crohn patiënten met de 3020insC-mutatie. De ziekte van Crohn wordt van oudsher geassocieerd met *M. paratuberculosis*, hoewel hierover veel controversie bestaat. In **hoofdstuk 10** tonen we aan dat *M. paratuberculosis* door TLR2, TLR4 en Nod2 wordt herkend. De cytokine productie door cellen van Crohn patiënten met de 30220insC mutatie was ernstig gestoord. Hoewel speculatief, deze resultaten passen in het concept dat een verstoorde afweer tegen intracellulaire pathogenen zoals *M. paratuberculosis* mogelijk een rol speelt in de pathogenese van de ziekte van Crohn.

In de voorgaande hoofdstukken hebben we laten zien dat er tussen TLRs en Nod2 een synergistische interactie bestaat als beide receptoren simultaan worden gestimuleerd op primair humane cellen. Dit model komt overeen met de simultane activatie van pathways tijdens een infectie. In de darm is er echter sprake van een continue expositie aan commensale micro-organismen en fragmenten van deze micro-organismen. Zo kan MDP inderdaad de mucosa van de darm passeren en aan worden getroffen in het weefsel. In **hoofdstuk 11** hebben we humane mononucleaire cellen gepreïncubeerd met MDP om de chronische stimulatie in de darm te simuleren, en vervolgens zijn deze cellen met TLR liganden en intestinale pathogenen gestimuleerd. Met deze experimenten laten we zien dat chronische stimulatie van Nod2 met MDP tot een verminderde TNF productie leidt na stimulatie van TLR4, terwijl de TLR2 pathway niet werd beïnvloed. Deze kruis-tolerantie voor TLR4-ligand kan een verklaring zijn waarom Nod2-mutaties leiden tot een chronische inflammatie van de darm in de ziekte van Crohn. In cellen met de 3020insC-mutatie is deze kruis-tolerantie inderdaad verdwenen en leidt dit tot een verhoogde productie van TNF zoals dit wordt gezien in de ziekte van Crohn. Het mechanisme hoe Nod2 een rol speelt in de pathogenese van de ziekte van Crohn is nog steeds controversieel: hoe kan een verhinderde herkenning van MDP door Nod2 leiden tot een pro-inflammatoire ziekte? Het verlies van kruis tolerantie zou een verklaring kunnen zijn. Anderzijds is de verminderde productie van anti-inflammatoire cytokine IL-10 met als gevolg een bias naar Th1-respons een ander mogelijk mechanisme. In beide modellen speelt de interactie van verschillend klasse PRRs een centrale rol en onderstreept het de immunomodulerende eigenschap van MDP door Nod2-herkenning.

In **hoofdstuk 12** hebben we de immunomodulerende effecten van andere muramylpeptiden onderzocht waarvan bekend is dat ze worden herkend door Nod1, een andere belangrijke NLR receptor. Muramyltripeptide (MTP) is de ligand voor Nod1 en geeft, net als MDP, een synergistische cytokine productie als MNC simultaan met TLR liganden worden gestimuleerd.

Daarbij viel op dat cellen met de 3020inC mutatie ook geen cytokine produceerde op MTP. Dit suggereert een gemeenschappelijke pathway voor Nod1 en Nod2 die door deze mutatie is aangedaan. De synergie tussen Nod1 en TLRs was echter niet aangedaan in deze cellen, dit betekent dat de *signal pathway* die tot deze interactie leidt, verschilt met de *pathway* die direct leidt tot cytokine inductie door Nod1 en Nod2. Er zijn verschillende studies die aantonen dat NLRs op verschillende niveaus functies hebben: 1. activeert *pathways* die direct tot NF-κB-activatie leiden en transcriptie van cytokines; 2. vormt complexen met andere moleculen om enzymen te activeren voor post-translationele modificatie van cytokines; 3. interfereert met ATP-signalering voor de secretie van cytokines; 4. reguleert transcriptiefactoren onafhankelijk van MDP. Al deze mechanismen onderstrepen de potentiële rol van NLRs in de modulatie van de immuun respons door interactie met vele *signal pathways* geactiveerd door TLR stimulatie.

Algemene conclusie

In mijn proefschrift toon ik aan dat er sprake is van een complexe interactie tussen verschillende klassen PRRs, dit leidt tot de inhibitie of versterking van de cytokinerespons geïnduceerd door micro-organismen en hun liganden. Deze immunomodulerende effecten spelen een rol in de afweerreactie tegen infecties (resistentie), maar ook in de homeostase tussen gastheer en commensale micro-organismen (tolerantie). Als deze interactie tussen PRRs is verstoord (door mutaties, blokkade door pathogenen of medicatie) kan dit op twee manieren tot ziekte leiden. In de eerste plaats kan een afgenomen immuunrespons ontstaan door de verminderde cytokine productie leiden tot een verhoogde vatbaarheid voor infecties. Ten tweede kan door een afgenomen demping van pro-inflammatoire cytokines er een verminderde tolerantie van commensale micro-organismen ontstaan en hierdoor chronische inflammatie. Het begrijpen van deze mechanismen zal belangrijk zijn voor het ontwikkelen van nieuwe therapeutische strategieën. Verbetering van resistentie tegen pathogenen door vaccinatie kan worden bereikt door verhoging van cytokineproductie die leidt tot een optimale cellulaire respons, zoals een Th1- en Th17-respons. Ontwikkeling van nieuwe adjuvantia gebaseerd op de synergie tussen verschillende PRR *signal pathways* kan hieraan bijdragen. Dit wordt onder andere geïllustreerd door CpG, een TLR9-agonist, die als adjuvans wordt gebruikt om de Th1 respons te stimuleren. Geactiveerde inflammatoire *signal pathways* door commensaal microbiële flora zouden kunnen worden gedempt waardoor er een relatieve toename van de anti-inflammatoire respons ontstaat die leidt tot de inductie van Th2 and Treg. Een deel van de effecten van probiotica en voedingssupplementen kan mogelijk aan dit mechanismen worden toegeschreven. Deze inzichten in de inductie van tolerantie door stimulatie van PRRs heeft veel aandacht in het medische onderzoek en kan mogelijk leiden tot nieuwe therapieën voor chronisch inflammatoire ziekten, zoals de ziekte van Crohn.
Concluderend kan men stellen dat de regulatie van de aangeboren afweer complex is, waarbij de interactie tussen de verschillende klassen receptoren centraal staat. In dit proefschrift hebben we geprobeerd een aantal aspecten van dit complexe netwerk te belichten, om zo een basis te leggen voor de ontwikkeling van nieuwe therapieën voor infectie- of auto-inflammatoire ziekten.

Dankwoord

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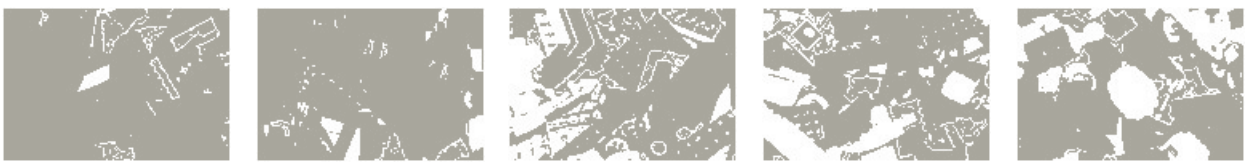

Jules Deelder. ik gedicht

Dankwoord

Nu eindelijk echt het laatste loodje, maar mijn dank betuigen is zeker niet zwaar. Met hulp van vele mensen is mijn proefschrift tot een heus boekje volbracht. Hierbij heeft het 'ik - gedicht' van Jules Deelder en mijn levensmotto mij telkens weer leren relativeren: ik maak me geen illusies, maar ik blijf optimist.

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ligt torenhoog, met minder geen genoegen. Beste Calin, Ik bewonder je enorme daadkracht en doorzettingsvermogen. Ik mocht jouw paranimf zijn en vond die dag echt geweldig, ik ben je dankbaar voor je vertrouwen in mij en je betrokkenheid. Ook wil ik Bart(je) op deze plek bedanken. Ik heb nu de gelegenheid om nogmaals te zeggen dat ik enorm trots op jou ben. De laatste jaren heb ik vaak moeten uitleggen dat niet ik de auteur was, maar jij. Sinds je je op een wetenschappelijke manier met evolutie bezighoudt, ben je gegroeid tot een top-wetenschapper en lijken alle puzzelstukjes (ook van je leven) een plek te hebben gekregen. Dit is nog maar het begin! Ze zullen vaker van ons horen. Dan wil ik nog Leo bedanken, het hoofd van dit geweldige lab. Wij hebben de laatste twee jaar intensiever samengewerkt. Je hebt het focus van het lab verruimd van innate naar adaptieve, van academisch naar toegepast (commercieel) en van infectie naar auto-immuun (en auto-inflammatoir). Ik hoop dat ik in de toekomst bij je binnen mag blijven lopen voor de ins and outs van het wetenschappelijk bedrijf.

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List of publications

List of Publications

The role of Toll-like receptors and C-type lectins for vaccination against *Candida albicans.* Ferwerda G, Netea MG, Joosten LA, van der Meer JW, Romani L, Kullberg BJ. *Vaccine*. 2009 Oct 31 (review)

Human dectin-1 deficiency and mucocutaneous fungal infections. Ferwerda B†, Ferwerda G†, Plantinga TS, Willment JA, van Spriel AB, Venselaar H, Elbers CC, Johnson MD, Cambi A, Huysamen C, Jacobs L, Jansen T, Verheijen K, Masthoff L, Morré SA, Vriend G, Williams DL, Perfect JR, Joosten LA, Wijmenga C, van der Meer JW, Adema GJ, Kullberg BJ, Brown GD, Netea MG. *N Engl J Med.* 2009 Oct 29;361(18):1760-7

Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, Funk CJ, Mason RJ, Kullberg BJ, Rubartelli A, van der Meer JW, Dinarello CA. *Blood.* 2009 Mar ;113(10):2324-35

Differential function of the NACHT-LRR (NLR) members Nod1 and Nod2 in arthritis. Joosten LA, Heinhuis B, Abdollahi-Roodsaz S, Ferwerda G, Lebourhis L, Philpott DJ, Nahori MA, Popa C, Morre SA, van der Meer JW, Girardin SE, Netea MG, van den Berg WB. *Proc Natl Acad Sci U S A.* 2008 Jul 1;105(26):9017-22

Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ. *Cell Microbiol.* 2008 Oct;10(10):2058-66

Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR, Kerrigan A, Tsoni SV, Gordon S, Meyer-Wentrup F, Adema GJ, Kullberg BJ, Schweighoffer E, Tybulewicz V, Mora-Montes HM, Gow NA, Williams DL, Netea MG, Brown GD. *Eur J Immunol.* 2008 Feb;38(2):500-6

Engagement of Nod2 has a dual effect on proIL-1beta mRNA transcription and secretion of bioactive IL-1beta. Ferwerda G, Kramer M, de Jong D, Piccini A, Joosten LA, Devesaginer I, Girardin SE, Adema GJ, van der Meer JW, Kullberg BJ, Rubartelli A, Netea MG. *Eur J Immunol.* 2008 Jan;38(1):184-91

Crohn's disease patients homozygous for the 3020insC Nod2 mutation have a defective NOD2/TLR4 cross-tolerance to intestinal stimuli. Kullberg BJ, Ferwerda G, de Jong DJ, Drenth JP, Joosten LA, Van der Meer JW, Netea MG. *Immunology.* 2008 Apr;123(4):600-5

Immune recognition of *Candida albicans* beta-glucan by dectin-1. Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, Walker L, Jansen T, Jacobs L, Tsoni V, Brown GD, Odds FC, Van der Meer JW, Brown AJ, Kullberg BJ. *J Infect Dis.* 2007 Nov 5;196(10):1565-71.

***Mycobacterium paratuberculosis* is recognized by Toll-like receptors and Nod2.** Ferwerda G, Kullberg BJ, de Jong DJ, Girardin SE, Langenberg DM, van Crevel R, Ottenhoff TH, Van der Meer JW, Netea MG. *J Leukoc Biol.* 2007 Oct;82(4):1011-8

Recognition of fungal pathogens by toll-like receptors. Netea MG, Ferwerda G, van der Graaf CA, Van der Meer JW, Kullberg BJ. Curr Pharm Des. 2006;12(32):4195-201 (review)

Normal responses to specific Nod1 ligands. Ferwerda G, Girardin SE, van der Meer JW, Netea MG. *Eur J Immunol.* 2006 Oct;36(10):2817-8 (letter)

Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. Netea MG, Azam T, Ferwerda G, Girardin SE, Kim SH, Dinarello CA. *J Leukoc Biol.* 2006

Defective acute inflammation in Crohn's disease. Netea MG, van der Meer JW, Ferwerda G, Kullberg BJ. *Lancet.* 2006 Aug 12;368(9535):577-8 (letter)

Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G, Hobson RP, Bertram G, Hughes HB, Jansen T, Jacobs L, Buurman ET, Gijzen K, Williams DL, Torensma R, McKinnon A, MacCallum DM, Odds FC, Van der Meer JW, Brown AJ, Kullberg BJ. *J Clin Invest.* 2006 Jun;116(6):1642-50

Nod2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis.* Ferwerda G, Girardin SE, Kullberg BJ, Le Bourhis L, de Jong DJ, Langenberg DM, van Crevel R, Adema GJ, Ottenhoff TH, Van der Meer JW, Netea MG. *PLoS Pathog.* 2005 Nov;1(3):279-85

IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. Netea MG, Azam T, Ferwerda G, Girardin SE, Walsh M, Park JS, Abraham E, Kim JM, Yoon DY, Dinarello CA, Kim KH. *Proc Natl Acad Sci U S A.* 2005 Nov 8;102(45):16309-14

Nod2 3020insC mutation and the pathogenesis of Crohn's disease: impaired IL-1beta production points to a loss-of-function phenotype. Netea MG, Ferwerda G, de Jong DJ, Girardin SE, Kullberg BJ, van der Meer JW. *Neth J Med.* 2005 Sep;63(8):305-8

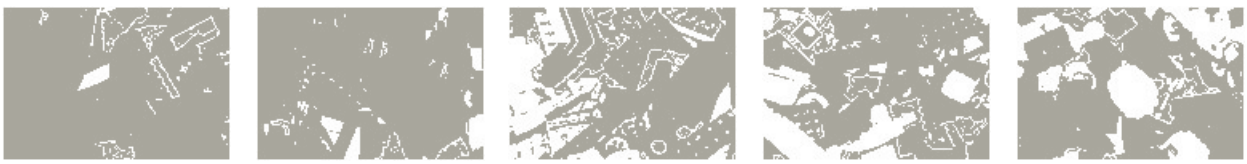
The frameshift mutation in Nod2 results in unresponsiveness not only to Nod2- but also Nod1-activating peptidoglycan agonists. Netea MG, Ferwerda G, de Jong DJ, Werts C, Boneca IG, Jéhanno M, Van Der Meer JW, Mengin-Lecreulx D, Sansonetti PJ, Philpott DJ, Dharancy S, Girardin SE. *J Biol Chem.* 2005 Oct 28;280(43):35859-67.

Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. Netea MG, Ferwerda G, de Jong DJ, Jansen T, Jacobs L, Kramer M, Naber TH, Drenth JP, Girardin SE, Kullberg BJ, Adema GJ, Van der Meer JW. *J Immunol.* 2005 May 15;174(10):6518-23.

Long term results of pneumatic dilation in achalasia followed for more than 5 years. West RL, Hirsch DP, Bartelsman JF, de Borst J, Ferwerda G, Tytgat GN, Boeckxstaens GE. Long term results of pneumatic dilation in achalasia followed for more than 5 years. *Am J Gastroenterol.* 2002 Jun;97(6):1346-51.

Nicotinic acetylcholine receptor blocking effect of guanethidine in the rat gastric fundus. Blommaart PJ, Ferwerda G, Kodde A, Tytgat GN, Boeckxstaens GE. *Br J Pharmacol.* 1999 Oct;128(4):903-8.

Curriculum Vitae



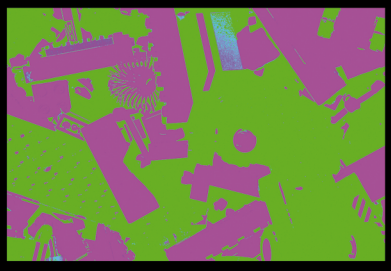
Curriculum Vitae

Jacob Gerben Ferwerda werd geboren op 15 juni 1973 te Haarlem. In 1990 haalde hij zijn HAVO diploma en in 1992 zijn VWO diploma aan het Eerste Christelijk Lyceum in Haarlem. Hierna heeft hij twee maanden in Ghana vrijwilligerswerk gedaan voor Voluntary Workcamps Association of Ghana (VOLU).

Vanaf 1992 studeerde hij geneeskunde aan de Vrije Universiteit in Amsterdam. Tijdens zijn studie heeft hij drie jaar gewerkt als studentenassistent fysiologie en is hij een jaar bestuurslid geweest van de Societas Studiosorum Reformatorum Amsterdam (SSRA). In 1997 haalde hij zijn doctoraal examen en in 2000 zijn arts examen (cum laude). Hierna heeft hij als arts-onderzoeker gewerkt op de afdeling gastro-enterologie van het Amsterdams Medisch Centrum. Eind 2000 is hij begonnen als arts niet in opleiding op de afdeling interne geneeskunde van het Onze Lieve Vrouwe Gasthuis in Amsterdam. In 2002 begon hij aan zijn opleiding interne geneeskunde in het Rijnstate Ziekenhuis te Arnhem (opleider dr. R van Leusen), vanaf 2004 is deze voortgezet in het Universitair Medisch Centrum St Radboud (opleiders prof. dr. PMJ Stuyt, dr. J de Graaf en prof. dr. JWM van der Meer). In augustus 2009 rondde hij zijn opleiding af en is hij geregistreerd als algemeen internist.

Tijdens zijn opleiding interne geneeskunde is hij in oktober 2004 gestart met zijn promotie-onderzoek bij prof. dr. BJ Kullberg, prof. dr. JWM van der Meer en prof. dr. MG Netea, gesubsidieerd door het NWO. Tijdens dit onderzoek heeft hij een half jaar gewerkt aan Oxford University in de Sir William Dunn School of Pathology op het laboratorium van Prof. dr. Siamon Gordon. Sinds september 2009, na het afronden van zijn opleiding interne geneeskunde is hij gestart als postdoc op het Laboratorium Kindergeneeskunde Infectieziekten (LKI) van het Universitair Medisch Centrum St Radboud onder leiding van Prof. dr. PW Hermans.

Hij trouwde in 2002 met Rosa Mengde, hun dochter Pien werd in 2003 geboren, hun zoon Lode in 2005 en hun dochter Saar in 2008.



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